UNIVERSIDADE ESTADUAL DE CAMPINAS

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PARTICIPAÇÃO DO ÓXIDO NÍTRICO NO DESENVOLVIMENTO DA ENCEFALOMIELITE ALÉRGICA EXPERIMENTAL EM RATOS

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aos meus pais Luiz e Maria

pelo carinho e total confiança

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"Você vem pra me proteger
nas horas mais duras,
Sua mão me segura
e me faz levantar..."

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SUMÁRIO

RESUMO

O óxido nítrico (NO) é um radical livre produzido endogenamente a partir da oxidação de um dos nitrogênios do grupo guanidino terminal do aminoácido L-arginina por oxigênio molecular. Essa biossíntese é catalisada por uma família de enzimas denominadas óxido nítrico sintases (NOS), as quais podem ser inibidas por análogos estruturais da L-arginina que possuem grupos substituintes no grupo guanidino terminal. O NO tem significativa reatividade com espécies químicas diversas tais como oxigênio, ânion superóxido, grupos tióis, metais de transição, etc; sendo que os íons nitrito (NO_2^-) e nitrato (NO_3^-) são os produtos finais estáveis da oxidação do NO no entorno fisiológico.

NO, e espécies reativas derivadas do nitrogênio (NDRS) em geral, participam de vários processos fisiológicos no cérebro, incluindo neurotransmissão, neuromodulação e plasticidade sináptica, assim como de processos patológicos tais como neurodegeneração e neuroinflamação. Altos níveis de NO têm sido associados com a atividade da encefalomielite alérgica experimental (EAE), modelo empregado amplamente em animais para o estudo da esclerose múltipla em humanos. Neste trabalho, nós caracterizamos a atividade e a expressão das isoformas das NOS, bem como a presença de resíduos protéicos de nitrotirosina (NT, como indicador da produção de peroxinitrito) em massa encefálica total (MET) de ratos Lewis durante o desenvolvimento da EAE. A EAE foi induzida em ratos Lewis através da administração subcutânea de uma emulsão contendo proteína básica de mielina (MBP) e a evolução clínica da doença (estágios 0 - III) foi avaliada diariamente. No final de cada estágio, as METs foram removidas, e análises da atividade de NOS, expressão de proteínas contendo resíduos de NT, e conteúdo de mRNA para as diferentes isoformas de NOS foram realizadas. Amostras de sangue também foram coletadas para a medida dos ânions NO_2^- e NO_3^- no soro, como índice da produção de NO sistêmico. Na falta de adição de qualquer cofator exógeno de NOS (exceção feita para o NADPH), a atividade de NOS dependente de Ca^{2+} foi significativamente diminuída nos ratos com EAE-III quando comparada com as dos grupos controle, EAE-0 ou EAE-I, mas nenhuma diferença foi observada entre os grupos na atividade de NOS independente de Ca^{2+}. Por outro lado, quando os cofatores calmodulina (CaM), FAD, tetrahidrobiopterina (BH4)

e NADPH foram adicionados, nenhuma diferença na atividade de NOS dependente de Ca^{2+} foi observada entre os grupos controle e EAE-III. Uma diminuição similar também foi observada na atividade de NOS dependente de Ca^{2+} dos grupos controle e EAE-III quando o FAD foi omitido do meio de incubação, mas nenhuma alteração ocorreu na ausência dos outros cofatores. Um aumento não significativo foi observado na atividade de NOS independente de Ca^{2+} da MET de ratos com EAE-III quando comparada à atividade obtida pelo grupo controle na presença de todos os cofatores. A atividade de NOS independente de Ca^{2+} de ratos com EAE-III não foi afetada pela omissão de qualquer um dos cofatores adicionados, enquanto que a do grupo controle foi inibida significativamente tanto pela ausência de FAD como de BH_4 no meio de incubação. A análise por RT-PCR não revelou qualquer alteração significativa no mRNA das isoformas nNOS ou eNOS entre os grupos experimentais; contudo, níveis aumentados de mRNA para iNOS foram detectados em homogenatos de MET de ratos com EAE-III em comparação com os animais controle. A análise de proteínas contendo resíduos de NT por Western blot mostrou um aumento significativo na intensidade de duas bandas (pesos moleculares 53 e 28 kDa) nos grupos EAE-II e EAE-III em comparação com o grupo controle. Os níveis de NO_3^- sérico foram encontrados significativamente mais altos nos ratos com EAE III do que nos animais controle. Homogenatos de MET ou soro de animais controle mostraram sobre a atividade de NOS exacerbado efeito inibitório quando comparado àquele observado para homogenatos de MET ou soro de animais com EAE-III. No entanto, as diferenças entre os grupos desaparecem após desnaturação por aquecimento (tanto dos homogenatos de MET como dos soros).

Com base nesses resultados, nós concluímos que paralelo a uma diminuição da atividade de NOS dependente de Ca^{2+}, um aumento da expressão ("up-regulation") de iNOS ocorre durante estágios mais avançados da doença, o qual poderia ser responsável pelo aumento da produção de NO sistêmico observado e ao aparecimento de proteínas modificadas por nitração de resíduos de tirosina a nível de sistema nervoso central. Por outro lado, a diminuição da sensibilidade de iNOS para ambos FAD e BH_4, e a ausência de inibidor circulante termolábil de NOS observado em animais com EAE reflete uma complexa modulação da atividade de NOS nos diferentes níveis de sítios regulatórios nesse modelo animal de esclerose múltipla.

ABSTRACT

Nitric oxide (NO) is a free radical endogenously synthetised by oxidation of one of the guanidinum nitrogen atoms of the aminoacid L-arginine by molecular oxygen. This biosynthesis is catalised by a family of enzimes generically known as nitric oxide synthases (NOS), which can be inhibited by structural analogs of L-arginine having substituent groups at the terminal guanidinum moeity. NO is extremely reactive toward different chemical species such as oxyigen, superoxide anion, thiol groups, transition metal ions, etc; nitrite (NO_2^-) and nitrate (NO_3^-) anions are the stable end products which result from NO oxidation within the physiological environment.

NO, and nitrogen-derived reactive species (NDRS) as a whole, take part of several physiological processes in the brain, including neurotransmision, neuromodulation and synaptic plasticity, as well as pathological processes such as neurodegenerative diseases and neuroinflammation. Increased nitric oxide (NO) production has been associated with disease activity in experimental allergic encephalomyelitis (EAE). In this study, we characterized the expression and activity of the different NOS isoforms, as well as the presence of protein nitrotyrosine residues (NT, as a marker of peroxynitrite production) in whole encephalic mass homogenates during the development of EAE in rats. EAE was induced in Lewis rats by subcutaneous injection of an emulsion containing myelin basic protein and the development of the disease (stages 0 - III) was clinically evaluated daily. In the end of the different stages, whole encephalic masses (WEM) were removed for further analysis of NOS activity, protein NT residues (Western blot) and mRNA for the different NOS isoforms (RT-PCR). Blood samples were also collected for measurement of serum NO_2^- and NO_3^- as an index of systemic NO production.

In the absence of the addition of any exogenous NOS cofactor (with the exception made for NADPH), Ca^{2+}-dependent NOS activity was significantly decreased in rats with EAE at stage III when compared with either control, EAE-0 or EAE-I groups, but no differences in Ca^{2+}-independent NOS activity were observed among the groups. In the other hand, when the cofactors calmodulin (CaM), FAD, tetrahydrobiopterin (BH_4) and NADPH were exogenously added, no differences in WEM Ca^{2+}-dependent NOS activity

were observed between control and EAE III groups. Similar decreases in Ca^{2+}-dependent NOS from either control or EAE III rats were observed when FAD was omitted from the incubation media, but no changes occurred in the absence of the other cofactors. A non-significant increase in Ca^{2+}-independent NOS activity was observed in WEM from EAE III rats compared to those from control animals in the presence of all the cofactors. Ca^{2+}-independent NOS activity in WEM homogenates from EAE III rats was not affected by the omission of any of the added cofactors, while the one from control animals was significantly inhibited by the absence of either FAD or BH_4 in the incubation media. RT-PCR analysis revealed no significant changes in either nNOS or eNOS mRNA among the groups of rats; however, increased levels of iNOS mRNA were detected in WEM homogenates from EAE III in comparison with those from control animals. Western blotting of NT-containing proteins showed two major bands (MW: 53 and 28 kDa) of significantly increased intensity at both stages II and III in comparison with controls. Serum NO_3^- levels were found significantly higher in EAE III rats than in control animals. The addition of either WEM homogenates or sera from control animals to a NOS preparation led to an exacerbated enzyme inhibition than that observed with WEM homogenates or sera obtained from EAE III rats. However, differences between groups disappear after heat-denaturation of both the WEM homogenates and sera.

Based on these results we conclude that parallel to a decrease of constitutive Ca^{2+}-dependent NOS activity, up-regulation of iNOS occurs during advanced EAE stages, which could be responsible for the observed increase in systemic NO production and the appearance of NT-modified proteins at the CNS level. On the other hand, the decreased sensitivity of iNOS to both FAD and BH_4, and the absence of a thermolabile circulating NOS inhibitor observed in animals with EAE reflect a complex modulation of NOS activity at different regulatory site levels in this animal model of multiple sclerosis.

LISTA DE ABREVIATURAS

AG:	Aminoguanidina
AMPc:	Adenosina monofosfato cíclica
AP:	Fosfatase alcalina
APC:	Células apresentadoras de antígenos
BCIP:	Fosfato de 5-bromo-4-cloro-3-indolil
BH_4:	Tetrahidrobiopterina
BHE:	Barreira hemato-encefálica
BSA:	Albumina de soro bovino
CaM:	Calmodulina
CFA:	Adjuvante completo de Freund
DEPC:	Dietil piro-carbonato
dNTP:	Deoxinucleotídeo trifosfato
DTT:	Ditiotreitol
EAE:	Encefalomielite alérgica experimental
EDRF:	Fator relaxante derivado do endotélio
EDTA:	Ácido etileno diamino tetra acético
EGTA:	Ácido etileno glicol- bis (β- amino etil éter) N, N, N', N'- tetra acético
EM:	Esclerose múltipla
eNOS:	NOS endotelial
EPR:	Ressonância paramagnética eletrônica
FAD:	Flavina adenina dinucleotídeo.
FMN:	Flavina adenina mononucleotídeo
GAPDH:	Gliceraldeído 3-fosfato desidrogenase
GC:	Guanilato ciclase

GMPc:	Monofosfato cíclico de guanosina
HPLC:	Cromatografia líquida de alta eficiência
HRP	Peroxidase de rabanete
ICAM-1:	Molécula de adesão celular intercelular-1
IFN-γ:	Interferon-γ
IgG	Imunoglobulina
IL:	Interleucina
iNOS:	NOS induzível
LFA-1:	Função linfocitária-1
L-MMA:	N-monometil-L-arginina
L-NA:	N-nitro-L-arginina
L-NAME:	N-nitro-L-arginina metil éster
L-NMA:	L-nitro metil arginina
LPS:	Lipopolissacarídeo
LTα:	Linfotoxina α
MAG:	Glicoproteína associada à mielina
MBP:	Proteína básica de mielina
MET:	Massa encefálica total
MHC-II:	Moléculas do complexo de histocompatibilidade principal classe II
MOG:	Glicoproteína de mielina do oligodendrócito
MPO:	Mieloperoxidase
NADPH:	Forma reduzida da nicotinamida difosfato
NBT:	"Nitro blue tetrazolium"
NIL:	N-(1-iminoetil) L-lisina
NMDA:	N-metil D-aspartato
nNOS:	NOS neuronal

NO:	Óxido nítrico
NOS:	Óxido nítrico sintase
NT:	3-nitrotirosina
PCR:	Reação em cadeia da polimerase
PLP:	Proteína de proteolipídeo
PMSF:	Fluoreto de fenil metil sulfonila
RCP:	Redutases do sistema de citocromo P450
RT:	Transcriptase reversa
SDS-PAGE:	Eletroforese em gel de poliacrilamida e Lauril sulfato de sódio
SNC:	Sistema nervoso central
SOD:	Superóxido dismutase
TAE:	Tampão tris-acetato de sódio-EDTA
Taq:	*Termus aquaticus*
TCR:	Receptores de linfócitos T
TEMED	N, N, N', N'- tetrametil-etileno-diamino
TGF-β:	Fator de crescimento tumoral-β
Th:	Células T auxiliares
TNF-α:	Fator de necrose tumoral-α
Tris:	Tris (hidróxi metil) amino etano
VCAM-1:	Molécula de adesão celular vascular-1
VLA-4:	Antígeno tardio-4

1. INTRODUÇÃO

1.1 O ÓXIDO NÍTRICO

O óxido nítrico (NO) é uma pequena molécula extremamente reativa e que tem importantes funções bioregulatórias. Embora o NO tenha sido primeiramente caracterizado há mais de 200 anos atrás por Joseph Priesley, sua importância como substância endógena surgiu somente ao longo dos anos '80 (Groves e Wang, 2000).

A descoberta da produção endógena de NO surgiu como conseqüência de trabalhos voltados ao estudo da resposta inflamatória e da reatividade vascular.

As primeiras evidências da produção endógena de NO surgiram com Tannenbaum e colaboradores (1978) através de experimentos demonstrando a produção do íon nitrato atribuída ao metabolismo microbiano intestinal. Contudo, trabalhos posteriores mostraram que ratos desprovidos de flora intestinal excretavam na urina quantidades de íon nitrato comparáveis às dos ratos controle (Green et al., 1981), e que esta excreção aumentava na presença de processos infecciosos causados por injeção de lipopolissacarídeo (LPS) de membrana de *Escherichia coli* (Wagner et al., 1983).

Stuher e Marletta (1985) e Hibbs e col. (1987a) observaram que a capacidade de macrófagos murinos, em cultura, de matar bactérias, fungos e células tumorais era dependente da presença do aminoácido L-arginina no meio de cultura, e que de forma paralela a essa atividade acumulava-se íon nitrito no meio. Hibbs e col. (1987b) observaram que o nitrito era produzido a partir do substrato L-arginina por uma L-arginina desaminase e que L-citrulina era formada como um co-produto. Concluíram então, que a atividade bactericida, fungicida e tumoricida dos macrófagos era devida ao nitrito formado.

Em 1980, Furchgott e Zawadzki em experimentos com preparações vasculares, demonstraram que um fator lábil liberado do endotélio era capaz de causar relaxamento do músculo liso vascular. Este fator foi denominado como "fator relaxante derivado do endotélio" (EDRF) que tem a capacidade de difundir nas células musculares lisas adjacentes e provocar o relaxamento do tecido através da ativação da guanilato ciclase (GC) solúvel e a conseqüente elevação dos níveis de

guanosina monofosfato cíclico (GMPc), semelhante ao mecanismo observado com os nitrovasodilatadores. Bolotina e col.(1994), demonstraram que o EDRF também é capaz de relaxar o músculo vascular na presença de azul de metileno (um inibidor da GC solúvel) por ativação dos canais de potássio dependentes de cálcio. A característica de antiagregante plaquetário descrita para os nitrovasodilatadores também foi demonstrada para o EDRF a partir de experimentos realizados *in vitro* (Radomski et al., 1987) e *in vivo* (Bhardwaj et al., 1988).

O EDRF foi química e farmacologicamente identificado como NO simultaneamente por Palmer e col. (1987) e Ignarro e col. (1987a, 1987b). Marletta e col. (1988) chegaram à mesma conclusão a respeito da origem dos íons nitrato e nitrito produzidos por macrófagos em cultura. De forma análoga às primeiras descobertas em culturas de macrófagos, o NO de origem endotelial foi confirmado como produto de oxidação da L-arginina (Palmer et al., 1988a,b).

A existência do NO no sistema nervoso central foi apontada pela primeira vez por Garthwaite e col. (1988). A confirmação veio após as observações de que análogos estruturais da L-arginina com substituintes no grupo guanidino aboliam o aumento de GMPc causado pela estimulação do glutamato via receptores do sub-tipo NMDA em cortes de cérebro (Bredt e Snyder, 1989; Garthwaite et al., 1989).

1.1.1 Síntese celular do óxido nítrico

O NO é produzido enzimaticamente por diversos tipos de células. As enzimas responsáveis por esta produção são genericamente chamadas de óxido nítrico sintases (NOS, E.C. 1.14.13.39) e existem sob diferentes isoformas.

Essas isoenzimas foram caracterizadas e purificadas de diferentes tipos de células e tecidos e possuem características bioquímicas, funcionais e mecanismos de regulação que as diferenciam. Todas se apresentam em forma de homodímeros onde cada monômero é constituído por dois domínios: um domínio de oxidase que contém sítios de ligação para o grupo heme, tetrahidrobiopterina (BH_4) e calmodulina (CaM) com o substrato L-arginina e um domínio de redutase com sítios

de ligação para a forma reduzida da nicotinamida difosfato (NADPH) e para as flavinas adenina mononucleotídeo (FMN) e dinucleotídeo (FAD).

Conforme Palmer e col. (1988a,b), o NO é produzido pela reação de oxidação de um dos nitrogênios do grupo guanidino terminal do substrato fisiológico L-arginina por oxigênio molecular (O_2, Leone et al., 1991). Esta oxidação envolve uma transferência de cinco elétrons e ocorre em duas etapas, conforme ilustrado na figura 1:

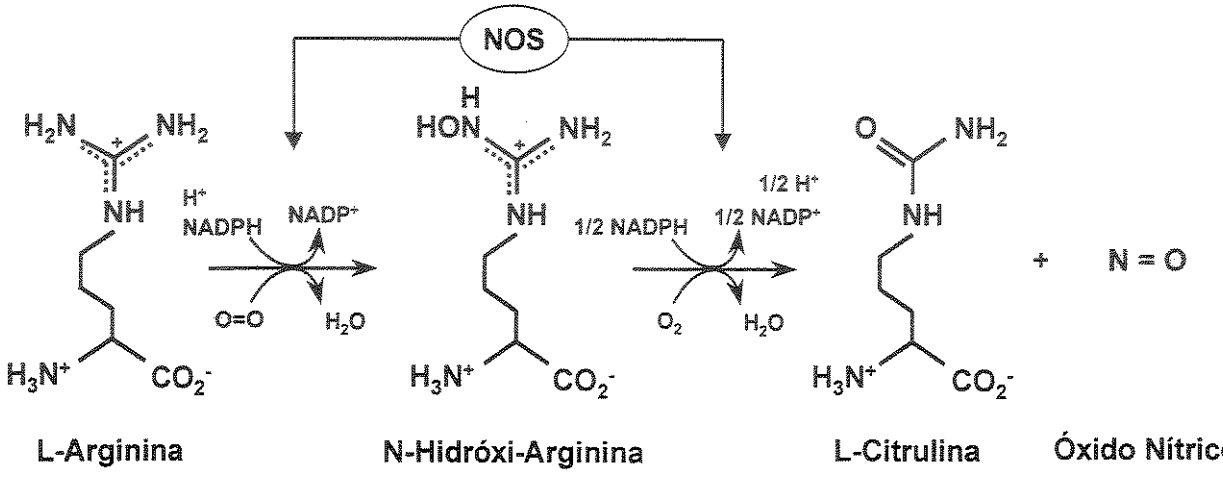


Figura 1. Representação esquemática das reações catalisadas pelas NOS (Groves e Wang, 2000).

Na primeira etapa forma-se, por transferência de dois elétrons, o produto intermediário N^G-hidróxi L-arginina (Stuher et al., 1991), o qual se mantém firmemente ligado à enzima; entretanto, sob determinadas condições, pode ser isolado como produto (Klatt et al., 1993). Esta primeira reação assemelha-se às clássicas oxidações mediadas pelas redutases do sistema de citocromo P450 (RCP) dependentes de NADPH. Nestas reações um equivalente eletrônico é transferido do NADPH à flavoproteína FAD, esta reduz o FMN, a qual, pela sua vez, reduz o Fe^{3+} do grupo heme (protoporfirina IX nas NOS de cérebro e macrófagos) para Fe^{2+}, conforme indicado na figura 2:

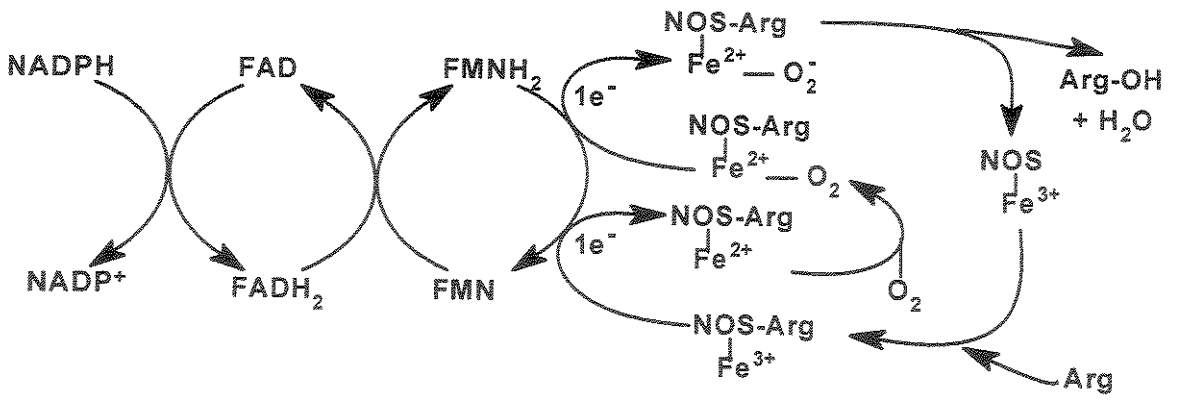


Figura 2. Representação esquemática das reações de óxido-redução envolvidas na síntese de NO endógeno.

O monóxido de carbono (CO) bloqueia esta etapa de hidroxilação, o qual é consistente com a participação de um grupo heme do tipo citocromo P450. O próprio NO parece exercer algum tipo de inibição por retroalimentação pelo mesmo mecanismo (Rengasamy e Johns, 1993; Assreuy et al., 1993). A reação é acelerada por BH_4 e requer CaM (Stuher et al., 1991; White e Marletta, 1992; Klatt et al., 1993). Estudos de atividade catalítica revelam que BH_4 é um dos cofatores mais importantes para a NOS, pois confere ação alostérica que estabiliza a estrutura dimérica de funcionamento da enzima, a conformação do grupo heme e a ligação do substrato L-arginina (Werner et al., 1998).

Na segunda etapa, ocorre a oxidação da N^G-hidróxi L-arginina a NO e L-citrulina pela transferência de mais três elétrons. Esta reação não está tão bem caracterizada quanto a primeira, já que qualquer mecanismo que tente explicá-la deverá dar conta dos seguintes achados experimentais: i) utiliza 0.5 equivalentes de NADPH; ii) requer O_2 e CaM; iii) é acelerada por H_4B; iv) é inibida por CO e análogos da L-arginina, mostrando, desta forma, comportamento farmacológico semelhante àquele observado na etapa de hidroxilação (Stuher et al., 1991; White e Marletta, 1992; Klatt et al., 1993). Modelos mecanísticos desta reação deveriam explicar o fato de que as duas reações de oxidação da L-arginina (sucessivas e independentes) ocorrem num sítio ativo comum da enzima (Bredt e Snyder, 1994).

1.1.2 Isoformas das NO sintases

De acordo com a ordem de isolamento e purificação, as diferentes isoformas de NOS têm sido nomeadas como NOS neuronal ou tipo I (nNOS), NOS induzíveis ou tipo II (iNOS, induzidas por citocinas e LPS), e NOS endotelial ou tipo III (eNOS).

Duas delas têm sido identificadas como constitutivas, ou seja, presentes em condições fisiológicas e reguladas pelo íon Ca^{2+}: a NOS neuronal, expressa no sistema nervoso central e periférico (Knowles and Moncada, 1994; Moncada e Higgs, 1995; Moncada et al., 1997) e a NOS endotelial, inicialmente detectada no endotélio vascular (Pollock et al., 1991; Mayer et al., 1991). A NOS induzível, expressa em situações patológicas em resposta à estímulos inflamatórios por algumas citocinas e outros compostos como LPS proveniente de bactérias, é independente de Ca^{2+} (NOS encontrada em macrófagos, hepatócitos, neutrófilos e em células do músculo liso; conforme revisão de Marín e Rodríguez-Martínez, 1997).

A figura 3, mostra um modelo esquemático dos sítios de reconhecimento dos cofatores para as diferentes isoformas de NOS e são comparadas com a redutase do citocromo P450 (RCP):

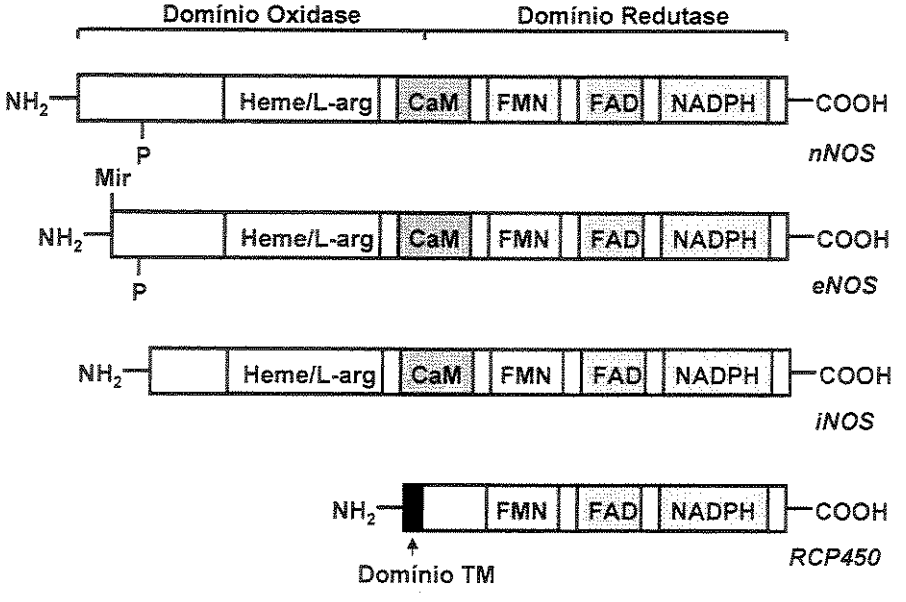


Figura 3. Representação esquemática dos sítios de reconhecimento dos cofatores para as diferentes isoformas de NOS comparadas com a redutase do citocromo P450 (Marin e Rodriguez Martinez, 1997).

NOS neuronal

Gorsky e col. (1989) foram os primeiros a relatar que a NOS presente em uma linha celular derivada de neuroblastoma era de localização citossólica, utilizava L-arginina como substrato, requeria NADPH como cofator, era regulada pelas concentrações fisiológicas intracelulares de Ca^{2+} e era dependente de CaM. Trabalhos posteriores determinaram que a NOS presente em homogenatos de cérebro total também era citossólica e que tinha os mesmos requerimentos que a enzima de neuroblastoma, sendo o cerebelo a área de maior atividade (Förstermann et al., 1990).

O isolamento desta isoforma (Bredt e Snyder, 1990) permitiu a posterior clonagem molecular (Bredt et al., 1991), e o cDNA codificava um polipeptídeo de 160 kDa que apresentava no seu extremo C-terminal (domínio de redutase) 36% de homologia com a RCP450 (vide Figura 3). Esta semelhança é mostrada por todas as isoformas de NOS clonadas até o presente e reflete o mecanismo oxidativo envolvido na biossíntese do NO. Na ausência de L-arginina, o domínio de redutase pode transferir elétrons do NADPH para o O_2, e formar assim ânion superóxido ($O_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2); estas espécies podem explicar as características neurotóxicas e neurodegenerativas dos aminoácidos excitatórios. Com base nestes fatos, Bredt e Snyder (1994) propõem que a NOS seja um resultado evolutivo da RCP.

Na Figura 3 mostra-se uma seqüência apta para fosforilação (P) da serina 372 mediada por uma proteína kinase dependente de AMPc (PKA). Curiosamente, foi demonstrado que o conteúdo de mRNA de nNOS em músculo esquelético de humanos é maior que no próprio cérebro, enquanto que no rato é quase indetectável (Nakane et al., 1993).

A nNOS também está expressa na medula dorsal (Dun et al., 1992), nas glândulas adrenais (Dun et al., 1993), em células epiteliais do útero, pulmão e estômago (Schmidt et al., 1992a), em plaquetas (Radomski et al., 1990) e células das ilhotas pancreáticas (Schmidt et al., 1992b)

NOS endotelial

Em 1989, Ishii e col. relataram que homogenatos de células endoteliais sintetizavam uma substância de características similares ao NO que elevavam os níveis intracelulares de GMPc em células RFL-6 (uma linha celular derivada de fibroblastos de pulmão de rato), e outros autores verificaram que a ^{3}H-L-arginina era convertida a ^{3}H-L-citrulina (Palmer e Moncada, 1989). Quando as frações intracelulares de células endoteliais em cultura foram analisadas, a atividade de NOS aparecia como 95% unida à fração particulada, e o restante 5% citossólica (Förstermann et al., 1991). A mesma distribuição foi encontrada em células endoteliais nativas (Mitchell et al., 1991). Através de eletroforese em SDS-PAGE foi demonstrado que o peso molecular da NOS particulada procedente tanto de células endoteliais nativas como em cultura é 135 kDa.

A eNOS particulada foi clonada e a seqüência predita pelo cDNA mostra 60% de homologia com a nNOS (Lamas et al., 1992; Sessa et al., 1992; Janssens et al., 1992). Existe alto grau de conservação entre os domínios para união de FAD, FMN, NADPH, CaM e de fosforilação (P) por PKA (vide Figura 3). Apesar da falta de domínio de localização transmembrana (TM), existe no extremo N-terminal uma seqüência para miristoilação (Mir), a qual explica a localização da proteína na fração particulada da célula endotelial. De fato, mutações nesta região dão como resultado a expressão de uma eNOS solúvel (Busconi e Michel, 1993).

NOS induzível

As observações de que mamíferos excretam íon nitrato na urina (Green et al., 1981b) e de que a injeção de ratos com lipopolissacarídeo (LPS) de parede de *Escherichia coli* aumenta a dita excreção (Wagner et al., 1983) estimularam à busca de células capazes de sintetizar nitratos. Em 1985, Stuehr e Marletta descobriram que macrófagos podiam ser induzidos com LPS a produzir quantidades significativas de íons nitrato e nitrito. Em 1987, Hibbs e col. sugeriram que esta produção era de origem enzimática e, no mesmo ano, Stuehr e Marletta obtiveram a mesma indução incubando macrófagos murinos com a citocina interferon-γ (IFN-γ).

A enzima responsável pela liberação de NO em macrófagos estimulados com LPS/IFN-γ (iNOS) foi encontrada na fração citossólica (Marletta et al., 1988) e posteriormente clonada (Xie et al., 1992; Lyons et al., 1992; Lowenstein et al., 1992).

O cDNA prediz uma seqüência de aminoácidos com 50% de identidade com a nNOS e 51% com a eNOS. O peso molecular é de 130 kDa. A região de fosforilação está ausente e o mRNA possui zonas de possível "splicing" (vide Figura 3, Xie et al., 1992).

O tratamento *in vitro* com LPS, IFN-γ, fator de necrose tumoral-α (TNF-α) e interleucina-1β (IL-1β) induz a síntese de NOS em hepatócitos (Geller et al., 1993), e a IL-1β a induz em condrócitos. Neutrófilos e monócitos circulantes de pacientes portadores de doença granulomatosa crônica tratados cronicamente com IFN-γ não mostraram liberação aumentada de NO (Condino-Neto et al., 1995).

O gene da iNOS apresenta no começo (promotor) duas regiões de regulação diferenciadas. A região I possui sítios de união para elementos relacionados ao LPS e a região II para fatores relacionados ao IFN-γ. Esta última não regula diretamente a expressão da NOS, mas amplifica o aumento codificado pela região I. Conclui-se assim, que os elementos responsivos ao LPS e ao IFN-γ atuam em duas regiões regulatórias diferentes do gene e que o LPS por si próprio é capaz de induzir a expressão da iNOS, enquanto que o IFN-γ só o faz na presença de LPS (Xie et al., 1993). Estas afirmações explicam resultados prévios a respeito da importância da seqüência de estimulação de macrófagos *in vitro* com LPS e IFN-γ na indução da NOS (Lorsbach e Russell, 1992).

Como foi anteriormente mencionado, a primeira etapa de oxidação da L-arginina a NO requer a união da CaM à NOS. No caso das isoformas constitutivas nNOS e eNOS, esta ativação só ocorre pela formação do complexo Ca^{2+}-CaM. De fato, a liberação de NO da célula endotelial promovido pela acetilcolina é decorrente da liberação dos estoques de Ca^{2+} intracelular pelo ciclo do fosfatidil inositol que segue à ativação dos receptores muscarínicos M_2 (Busse e Mülsch, 1990). O glutamato agindo nos receptores NMDA provoca um influxo de Ca^{2+} que, após união à CaM,

ativa a nNOS (Garthwaite et al., 1988). No caso das NOS induzíveis (provenientes de macrófagos, hepatócitos, etc.) a atividade não é estimulada por Ca^{2+} nem bloqueada por antagonistas de CaM. Nathan (1992) demonstrou que a CaM está estritamente unida às NOS induzíveis, e que essa união não é afetada pelo Ca^{2+}; no entanto a CaM não se une às NOS constitutivas a menos que o Ca^{2+} esteja presente.

1.1.3 Reatividade do óxido nítrico

O NO é uma molécula diatômica considerada um radical livre. No entanto, suas propriedades químicas são bem diferentes das de outras espécies radicalares como do radical hidroxila ($^{\bullet}OH$) e do ânion superóxido ($O_2^{\bullet-}$), do peróxido de hidrogênio (H_2O_2) e outras, chegando-se a considerar (no sentido estritamente químico) o NO como bastante inerte (Stamler et al., 1992).

Um certo número de compostos contém o grupo N-O e podem ser considerados como reservatórios de NO. Devido à estrutura da molécula, o NO faz parte dessas estruturas como íon positivo (NO^+), por perda de um elétron (nitrosônio) ou como íon negativo (NO^-), por ganho de um elétron (nitrosilo). Esse par redox (ou o seu ácido conjugado HNO) possui alvos que interagem no sistema biológico (Stamler et al., 1992). Sua função biológica e seu potencial de ação são determinados de acordo com as suas propriedades físicas e químicas. Por ser uma molécula de baixo peso molecular e razoavelmente hidrofóbica é altamente difundível. O NO é capaz de alcançar qualquer lugar dentro das células e tecidos (Feldman et al., 1993)

O maior fator limitante do NO é o ânion superóxido devido sua rápida interação (Stamler et al., 1992). Outras interações melhor caracterizadas incluem a reação com o oxigênio, interação com metais de transição como o ferro do grupo heme da hemoglobina, grupos aminas, tióis e aromáticos de proteínas e, dimerização.

Oxigênio

A toxicidade do NO é bem conhecida devido à sua grande reatividade com o oxigênio molecular. Esta reação resulta na formação de espécies altamente

oxidantes tais como o NO_2 (reação ***a***). O NO_2 dimeriza-se rendendo N_2O_4 (reação ***b***), e por sucessivas reações de dismutação (reação ***c***) e hidratação, forma os ácidos nitroso (HNO_2) e nítrico (HNO_3) em equilíbrio com os respectivos ânions nitrito (NO_2^-) e nitrato (NO_3^-; reações ***d*** e ***e*** da figura 4.

a) $2\,NO + O_2 \longrightarrow 2\,NO_2$

b) $2\,NO_2 \longrightarrow N_2O_4$

c) $2\,N_2O_4 \longrightarrow N_2O_3 + N_2O_5$

d) $N_2O_3 + H_2O \longrightarrow 2\,HNO_2 \rightleftharpoons 2\,NO_2^- + 2\,H^+$

e) $N_2O_5 + H_2O \longrightarrow 2\,HNO_3 \rightleftharpoons 2\,NO_3^- + 2\,H^+$

Figura 4. Esquema representativo das reações de formação de espécies reativas de nitrogênio e oxigênio (modificado de Marletta et al., 1988).

Experimentos realizados em fase aquosa, empregando um sensor eletroquímico específico para o NO, demonstram que as moléculas reagem de forma complexa, seguindo uma cinética de ordem zero para o NO (a diferença da ordem dois de fase gasosa) e de primeira ordem para o O_2 (Taha et al., 1992). Desta forma, a meia vida do NO (na concentração de 10 μM) na presença de 50 μM de O_2 foi de 4 minutos, a diferença de meia vida observada em sistemas biológicos está entre 3 e 30 segundos (Palmer et al., 1987, Myers et al., 1990).

Agrupamentos -NH_2 e SH

Outros importantes tipos de reações são os adutos que o NO forma com nucleófilos tais como aminas e grupos tiol.

A formação de N-nitrosaminas e diazoquinonas mutagênicas *in vivo* (Wu et al., 1993) tem sido demonstrada a partir da reação do NO com aminas secundárias e compostos fenólicos, respectivamente. Interessantemente, o ácido ascórbico inibe a

formação destes compostos *in vivo*, mas o mecanismo de ação deste antioxidante é questionável (Mirvish, 1993).

Reações de S-nitrosilação promovem a queda da atividade da enzima gliceraldeido-3-fosfato desidrogenase hepática observada em modelos de inflamação crônica (Molina y Vedia et al.,1992), ativação de plasminogênio sem perda das funções fibrinolíticas (Stamler et al., 1992), propriedades de antiagregante plaquetário e vasodilatador vascular por aumento do GMPc nas plaquetas e no músculo liso vascular respectivamente.

Os mecanismos de S-nitrosilação, recentemente encontrados, têm mostrado envolver a ceruloplasmina na oxidação de um elétron do NO. A ceruloplasmina é a maior proteína que contém cobre em sua estrutura e é encontrada no plasma dos mamíferos e sintetizada principalmente por hepatócitos, mas que pode ser expressa em macrófagos e astrócitos. Quando um composto é S-nitrosilado, ele funciona como um transportador de NO no sistema biológico, sendo o NO transferido para diferentes sulfidrilas de várias biomoléculas. Esta transferência pode ser mediada por reações de transnitração que ocorrem na presença de enzimas específicas denominadas dissulfeto isomerases (Akaike, 2000).

Proteínas com grupos tiol reduzidos ou mesmo compostos de baixo peso molecular como o aminoácido cisteína (Myers et al., 1990) ou a glutationa reduzida (Park et al., 1993) podem ser S-nitrosiladas e capazes de liberar o NO de forma lenta e espontânea (meias vidas de minutos a horas).

Metais de transição

A coordenação do NO com metais de transição como manganês, cobre ou ferro presentes em proteínas dá origem a sinais detectáveis por técnicas de ressonância paramagnética eletrônica (EPR). Esta tecnologia permitiu a interpretação molecular de muitas das ações biológicas do NO. Estes metais podem fazer parte de grupos heme ou agrupamentos metal-enxofre (principalmente Fe-S) presentes nas metaloproteínas.

A ativação da GC citossólica pelo NO é dependente do grupo heme. O Fe^{+2} está fracamente unido à porfirina e o NO tem grande afinidade pelo metal. Essa interação provoca o deslocamento do Fe^{2+} para fora do plano da porfirina. Esta mudança configuracional da enzima leva a um aumento tanto da velocidade máxima como da afinidade da GC pelo complexo Mg^{2+}-GTP, resultando na hidrólise para cGMP. A enzima retorna à forma inativa quando o Fe^{2+} deixa de ser complexado pelo NO e volta ao plano protoporfirina IX (Ignarro, 1992).

O NO interage com a hemoglobina (Hb) produzindo tanto NO-Hb como metahemoglobina (o NO-Hb reage com O_2, produzindo posteriormente metahemoglobina; Kosaka et al., 1989). A constante de velocidade de associação da Hb com o NO é similar àquela para o O_2 (aproximadamente 5×10^7 M^{-1} s^{-1}), mas as velocidades de dissociação são muito diferentes (aproximadamente 10^{-5} s^{-1} para o NO e 20 s^{-1} para o O_2; Stamler et al., 1992).

A síntese de diversos adutos do NO com agrupamentos Fe-S levou a compostos que possuem atividade vasodilatadora por ativação da GC solúvel (Flitney et al., 1990, 1992). Estes tipos de complexos foram detectados por espectrometria EPR em vários sistemas biológicos. Por exemplo, hepatócitos de rato em cultura expostos a diferentes estímulos inflamatórios, NO ou nitroprussiato de sódio mostraram sinais característicos do complexo Fe-NO (Stadler et al., 1993). Estes sinais resultam da destruição dos centro Fe-S pelo NO e são diferentes dos observados nos complexos formados com o grupo heme.

A destruição dos centros protéicos pelo NO foi assinalado como fator responsável pela rejeição ao transplante de coração em ratos. Estes adutos foram detectados por EPR tanto no sangue dos animais como no sítio da rejeição (Lancaster Jr et al., 1992).

O NO pode, pelo mesmo mecanismo, inativar enzimas mitocondriais envolvidas na cadeia respiratória (complexos I e II) e no ciclo dos ácidos tricarboxílicos (aconitase), e proteínas responsáveis pelo metabolismo do ferro tais como ferritina, transferrina e fatores reguladores do ferro a nível de mRNA (para revisão vide Henry et al., 1993).

Aceptores diversos do NO

Estruturas ressonantes de natureza química diversa também podem atuar como aceptores de NO.

A enzima ribonucleotídeo redutase é essencial na síntese de DNA. Por espectrometria de EPR foi demonstrado que no sítio ativo da subunidade R2 a enzima apresenta um radical livre tirosilo que é inativado pelo NO proveniente de macrófagos estimulados com LPS e IFN-γ (Lepoivre et al., 1994). É possível que este mecanismo, entre outros, seja o responsável pelas atividades anti-virais do IFN-γ.

Ânion superóxido

O ânion superóxido ($O_2^{\bullet}$) é formado a partir da redução do oxigênio molecular por transferência de um elétron. Pode ser formado como subproduto da cadeia respiratória mitocondrial gerado por autoxidação de flavoproteínas (Smith et al., 1997) ou durante o burst respiratório de fagócitos através da ativação da NADPH-oxidase (Kroncke et al., 1997).

A pH fisiológico, o potencial de oxidação do $O_2^{\bullet}$ é pequeno para oxidar outras moléculas, uma vez que requer uma segunda carga negativa (Sawyer e Valentine, 1981; Baum, 1984). A neutralização da primeira carga negativa por protonação facilita a ação do $O_2^{\bullet}$ como oxidante (pKa = 4,8; Bielski, 1978). Assim, o $O_2^{\bullet}$ age como oxidante somente em meio mais ácido ou quando a molécula atacada é um bom doador de elétrons como o ferro reduzido na forma de ligações Fe(II)-S. O Fe(III) é prontamente reduzido pelo $O_2^{\bullet}$ e essa habilidade de promover a redução de Fe(III) no citocromo c serve como a base para um ensaio usado freqüentemente para a quantificação da produção de $O_2^{\bullet}$ em sistemas biológicos (McCord e Fridovich, 1969).

Desde antes do descobrimento do NO como biomolécula e do estabelecimento das reações descritas acima, Gryglewski e col. (1986) e Rubanyi e Vanhoutte (1986) predisseram a natureza altamente reativa do EDRF com o $O_2^{\bullet}$ a partir das

observações de que a superóxido dismutase (SOD) potenciava a resposta vasorrelaxante de tais fatores.

Blough e Zafiriou (1985) foram os primeiros a relatar a formação do ânion peroxinitrito ($ONOO^-$) a partir da reação do NO com o $O_2^{\bullet}$ em soluções alcalinas. Em sistemas biológicos esta reação segue uma cinética de segunda ordem ($k = 4,6$ a $6,7 \times 10^9\ M^{-1}s^{-1}$; Huie e Padjama, 1993; Murphy et al.,1998, Saha et al., 1998) e é importante ressaltar que essa reação é três vezes mais rápida que a reação do $O_2^{\bullet}$ com a SOD (Klug et al., 1972). Isso implica em que o NO é o único alvo que o $O_2^{\bullet}$ reage mais rápido e que pode existir numa concentração suficiente para competir com a SOD. O $ONOO^-$ decompõe-se rapidamente nos radicais $NO_2^{\bullet}$ e $HO^{\bullet}$ (meia vida de 1,9 seg; Beckman et al., 1990):

$$NO^{\bullet} + O_2^{-\bullet} \longrightarrow ONOO^- + H^+ \rightleftharpoons ONOOH$$

$$ONOOH \longrightarrow HO^{\bullet} + NO_2^{\bullet} \longrightarrow NO_2^- + H^+$$

Uma série de células como as endoteliais, leucócitos, neutrófilos, neurônios e outras produzem ambos NO e $O_2^{\bullet}$, sendo que a facilidade de se combinarem é muito alta. O $ONOO^-$ mostra ter uma função nos processos celulares normais, impedindo que o $O_2^{\bullet}$ seja convertido a H_2O_2, no entanto, causa a oxidação de uma variedade de tipos de biomoléculas com conseqüências patológicas (Pryor e Squadrito, 1995).

O $ONOO^-$ reage de forma complexa com diferentes biomoléculas. Estas reações têm sido mostradas por peroxidação lipídica, como causa na quebra da fita de DNA, oxidação de resíduos de cisteína, lisina, metionina, e histidina, na nitração de compostos heterocíclicos como triptofano e guanina ou fenólicos como a tirosina (Demiryurek et al., 1998; Kelm et al., 1997; Koppenol et al., 1992).

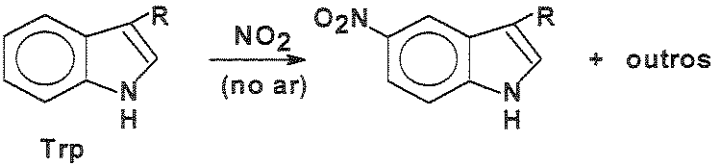
Também o $ONOO^-$ tem uma alta reatividade com o CO_2, em meio aquoso, levando a formação de espécies nitrantes mais eficientes que o próprio $ONOO^-$ (Squadrito e Pryor, 1998). O CO_2 é uma molécula unipresente nos tecidos e então sua reação com o $ONOO^-$ ocorre em uma larga fração da reatividade do NO *in vivo*.

No SNC, essa reatividade pode ser um fator pelo qual aumenta-se a nitração de resíduos de tirosina particularmente em patologias pelo qual se promove a acidose.

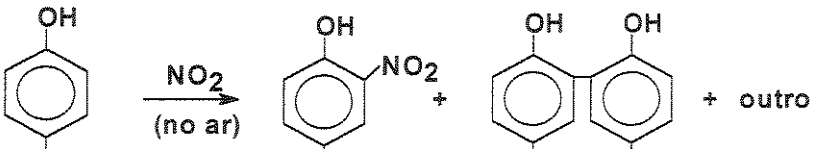
Com relação à reação de nitração do resíduos protéicos de tirosina que leva à formação da 3-nitrotirosina, esta pode causar danos em vários mecanismos como i) alteração na sinalização dependente de fosforilação de tirosina, visto a semelhança da nitrotirosina com a fosfotirosina, podendo assim bloquear de forma irreversível a fosforilação desta; ii) modificação na conformação de proteínas e aumento da proteólise pela adição de uma carga negativa na tirosina hidrofóbica e, iii) início de um processo autoimune, visto que nitrofenóis são considerados altamente antigênicos (Bartosz, 1996, ver revisão Torreilles et al., 1999).

Ao longo dos últimos anos, a presença de resíduos nitrosados de tirosina (3-nitrotirosina) livres ou em proteínas vem sendo considerada como indicadora da produção de ânion peroxinitrito *in vivo*. O desenvolvimento de anticorpos específicos anti-nitrotirosina permitiu aprofundar o conhecimento sobre a formação de $ONOO^-$ *in vivo* (Szabó et al., 1995).

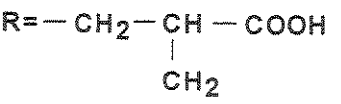
Independente do ânion peroxinitrito, Kikugawa e col. (1994) demonstraram que o radical NO_2 (toxina presente no ar poluído) quando exposto no ar (30 - 90 ppm) é capaz de reagir a pH 7.5 com os aminoácidos tirosina (Tyr) e triptofano (Trp) *in vitro:*



Trp



Tyr 3-nitro-Tyr diTyr



Estas mesmas reações tiveram lugar quando os aminoácidos faziam parte de proteínas tais como albumina bovina e gamma-globulinas humana. Van der Vliet e col. (1994) observaram também a formação de nitro-fenilalanina, *p, m* e *o*-tirosina a partir da reação da fenilalanina com ânion peroxinitrito *in vitro*, e demonstraram que a formação das tirosinas era decorrente da produção de radical $^{\bullet}OH$ a partir do peroxinitrito. Desta forma, sugerem que o ácido peroxinitroso possa sofrer uma ruptura homolítica originando os radicais $^{\bullet}OH$ e NO_2. Em trabalho posterior, o mesmo grupo propõe que o mecanismo de nitração da tirosina envolve a formação de um intermediário radicalar da tirosina (radical tirosil) e NO_2 proveniente do ânion peroxinitrito (Van der Vliet et al., 1995).

Baseados em considerações cinéticas, Beckman e col. (1994a) desqualificam a hipótese da nitração de resíduos protéicos de tirosina pelo NO_2 *in vivo*, e apontam o íon nitrônio (NO_2^+) como agente responsável por esta reação. O mecanismo de nitração de compostos aromáticos pelo NO_2^+ é bem conhecido e de ampla aplicação em química orgânica. Em trabalho anterior, o mesmo grupo de pesquisadores observou que a formação de 3-nitrotirosina pelo ânion peroxinitrito era catalisada pela enzima superóxido dismutase (SOD; Ischiropoulos et al., 1992), e sugeriram que o sítio ativo da enzima estaria envolvido na geração do íon nitrônio a partir do ânion peroxinitrito por mecanismo análogo ao da dismutação do ânion superóxido. Entretanto, na ausência de SOD, estes autores não justificam nem sugerem qualquer mecanismo de produção de íon nitrônio *in vivo* a partir do ânion peroxinitrito.

Estudos recentes mostram que a enzima mieloperoxidase (MPO) pode, em algumas circunstâncias, induzir reações de nitração de tirosina utilizando nitrito (NO_2^-) e ácido hipocloroso (HOCl) através da formação do cloreto de nitrila ($Cl\text{-}NO_2$; Kono et al., 1995; Eiserich et al., 1996; Panasenko et al., 1997; Patel et al., 1999), ocorrendo também nesse caso a cloração da tirosina.

Independente do(s) verdadeiro(s) mecanismo(s) de reação envolvido(s), a detecção de resíduos de tirosina nitrados em proteínas (ou livre) é indicadora da produção de ânion peroxinitrito *in vivo*. A ocorrência destas proteínas nitradas foi

relatada em diversos processos patológicos tais como modelos animais de endotexemia (Szabó et al., 1995), pacientes com artrite reumatóidea (Kaur e Halliwell, 1994) ou arterioscleróticos (Beckman et al., 1994b). Anticorpos anti-nitrotirosina tem se demonstrado ferramentas extremamente úteis na investigação das determinações da função de reações de nitração em patofisiologias dependentes do NO.

A presença do $ONOO^-$ em doenças neurodegenerativas tem sido relevante. Estudos *in vitro* demonstram sua toxicidade em cultura de células tais como de timócitos de ratos (Salgo et al., 1995 a, b), de neurônios (com o $ONOO^-$ agindo como inibidor da captação de glutamato; Trotti et al., 1996, 1998) e de uma linhagem de células PC12 resultando em necrose ou apoptose (Estévez et al., 1995).

O $ONOO^-$ tem sido sugerido contribuir em danos teciduais observados na esclerose lateral amiotrófica (Strong et al., 1998), esclerose múltipla (Cross et al., 1997), doença de Alzheimer (Smith et al., 1997 ; Good et al., 1996; Su et al., 1997) e doença de Parkinson (Ara et al., 1998; Beal, 1998).

1.2 A ESCLEROSE MÚLTIPLA

A esclerose múltipla (EM) é uma doença crônica inflamatória do SNC, caracterizada pela infiltração de células do sistema imune neste compartimento, destruição localizada de mielina e perda de oligodendrócitos (Ewing e Bernard, 1998). Embora sua etiologia ainda seja desconhecida, vários estudos clínicos, histopatológicos e genéticos indicam que a lesão do tecido é resultado da resposta imune voltada contra os constituintes da mielina (Brosnan e Raine, 1996).

Sua manifestação ocorre principalmente em adultos jovens. Os primeiros sintomas aparecem entre os 20 e 45 anos, raramente manifestando-se antes dos 15 anos ou após os 50 anos, embora existam relatos sobre a doença em crianças e idosos (Lynch e Rose, 1996).

A EM é caracterizada por disfunção neurológica, seguida por períodos de estabilização ou melhora. Clinicamente, durante os surtos, os pacientes podem apresentar vertigem, tremores, falta de coordenação, espasticidade aumentada, depressão, oscilação de humor, anormalidades cognitivas, disfunção sexual, ataxia, constipação intestinal, incontinência urinária, fadiga, neurite óptica, quadriparesia, disartria, perda de coordenação das extremidades superiores disestasia (Lynch e Rose, 1996).

De acordo com o desenvolvimento da doença, a EM pode ser classificada como benigna, de surtos e remissões ou como progressiva primária (Lynch e Rose, 1996).

A *EM benigna* ocorre mais frequentemente em mulheres jovens e os sintomas são ligeiros e normalmente sensoriais. Os problemas neurológicos são quase completamente resolvidos.

A *EM marcada por surtos e remissões* é a forma mais freqüente da doença. É caracterizada por períodos de disfunção neurológica (exacerbação, relapso ou ataque) seguida por períodos de estabilização ou melhora (remissão). Durante os períodos de remissões nem todos os sintomas são resolvidos completamente. Cerca de 30 a 50% dos pacientes pioram gradualmente e os sintomas e sinais neurológicos são acumulados. Essa forma é classificada como *EM progressiva crônica secundária*.

A *EM progressiva primária* é caracterizada por uma progressão de anormalidades neurológicas lenta. Os problemas mais comuns que pioram com o tempo incluem pariparesia espástica, ataxia cerebelar e incontinência urinária.

A patologia da EM é caracterizada pela presença de placas desmilielinizadas com inflamação perivascular, restritas à substância branca do SNC (Lynch e Rose, 1996). Em todas as lesões há predomínio de linfócitos e macrófagos, mas a extensão da destruição de oligodendrócitos depende do estágio da lesão, que pode ser ativa inicial, ativa tardia ou crônica. Embora sejam observados axônios intactos nas placas desmielinizadas, o dano axonal pode ser inferido pela presença de precursor de proteína amilóide, observada nas bordas ativas da lesão, que coincide

com a localização de macrófagos no local, sugerindo que a reação imuno-mediada não está restrita à mielina (Amor et al., 1997).

Na EM os contínuos ataques do sistema imune ao SNC, constituídos de períodos de surtos e remissões, reduzem a capacidade de remielinização das lesões, resultando na formação de placas permanentes de tecido cicatricial glial. Além disso, há redução no número de neurônios e conseqüentes deficiências neurológicas irreversíveis (Tourbah et al., 1998).

Embora os antígenos específicos, responsáveis pela resposta autoimune na EM não tenham sido totalmente determinados, postula-se que esse processo seja iniciado quando macrófagos que expressam moléculas do complexo de histocompatibilidade principal classe II (MHC-II) apresentem peptídeos antigênicos próprios modificados aos receptores de linfócitos T (TCR) nas células T auxiliares 1 (Th1). Durante a apresentação do antígeno, tem particular importância a presença de moléculas co-estimulatórias como a B7 e CD28. Posteriormente, leucócitos circulantes da vasculatura cerebral ligam-se fracamente ao endotélio através de moléculas de adesão, como o antígeno tardio-4 (VLA-4) e antígeno associado à função linfocitária-1 (LFA-1). Estas moléculas ligam-se de forma complementar à molécula de adesão celular vascular-1 (VCAM-1) e à molécula de adesão celular intercelular-1 (ICAM-1) na superfície das células endoteliais. A liberação de proteases pelos leucócitos lesam o tecido e facilitam a diapedese dessas células para o parênquima cerebral. Desta forma, a destruição da mielina seria mediada por células T que secretariam o fator de necrose tumoral-α (TNF-α), tóxico para oligodendrócitos e por macrófagos que a fagocitam. Os macrófagos também liberam TNF-α, radicais livres derivados do oxigênio, óxido nítrico (NO) e proteases. A destruição da mielina ocorre ainda pela ação de linfócitos B que diferenciam-se em plasmócitos, secretando anticorpos que ativam o sistema complemento, promovendo o ataque à bainha de mielina (Andersson e Goodkin, 1998).

A compreensão destes mecanismos é importante para o esclarecimento da patogênese de doenças autoimunes como a EM e formulação de estratégias terapêuticas para intervenção imune. Neste sentido, o emprego do modelo

experimental da EM, a encefalomielite autoimune (ou alérgica) experimental (EAE), tem trazido grandes contribuições (Ewing e Bernard, 1998).

A primeira descrição da EAE foi feita por Rivers e col. (1933). Esses autores demonstraram que a encefalomielite aguda disseminada, que ocasionalmente ocorre em pacientes após vacinação contra raiva, poderia ser reproduzida em macacos por repetidas injeções de extrato de cérebro de coelho (Macedo et al., 1996).

Em meados de 1940, vários autores observaram que quando antígenos do SNC eram emulsificados em adjuvante completo de Freund (CFA, micobactérias mortas em uma emulsão de óleo em água), e injetados em animais, já reproduzível em roedores, esses desenvolviam uma encefalomielite aguda dentro de poucas semanas, agora conhecida como EAE ativa (Morgan, 1946; Kabat et al., 1946).

Em 1960, Paterson demonstrou que EAE pode ser induzida de forma passiva pela injeção de linfócitos. A partir daí, essa forma de transferência tem sido feita pela injeção de células T encefalitogênicas ativadas, obtidas de animais sensibilizados.

A EAE pode ser induzida em várias linhagens animais suceptíveis à doença empregando-se imunização com o vírus da encefalomielite murina de Theiler, com o homogeneizado de SNC, ou com vários antígenos do SNC como a proteína básica de mielina (MBP), proteína de proteolipídeo (PLP), glicoproteína associada à mielina (MAG), glicoproteína de mielina do oligodendrócito (MOG) e gangliosídeos, ou ainda, por fragmentos purificados dessas proteínas emulsificados em CFA. Também pode ser induzida pela transferência passiva ou ativa de células T reativas à MBP ou PLP (Tsunoda e Fujinami, 1996).

Da mesma forma que na EM, o curso clínico da EAE pode ser igualmente variável. De acordo com o protocolo de indução aplicado, a doença pode apresentar-se sob a forma monofásica aguda ou sob a forma crônica com ataques e remissões.

A forma monofásica aguda pode ser induzida em ratos Lewis por imunização ativa com autoantígenos purificados de mielina em CFA ou por transferência ativa.

Em ambos os casos a doença é caracterizada por uma doença paralisante ascendente, que primeiro afeta a cauda e os membros posteriores, progredindo para os membros anteriores, levando eventualmente à morte. O exame histopatológico demonstra que a doença clínica na forma monofásica aguda está associada à inflamação do parênquima perivascular (Wekerle et al., 1994).

A indução de forma ativa consiste de duas fases: uma fase de indução e de uma fase efetora. Como demonstrado na Figura 5, a fase de indução envolve (1) a apresentação de epítopos da mielina aos linfócitos T CD4+ nos órgãos linfóides periféricos e (2) subseqüente expansão e diferenciação destas em células efetoras Th1, que secretam citocinas pró-inflamatórias. A fase efetora então, consiste na (3) migração de linfócitos mielina-específicos ativados para o SNC, levando à quebra da barreira hematoencefálica (BHE). Nesse momento, ocorre (4) a apresentação de epítopos da mielina por células apresentadoras de antígenos (APC) aos linfócitos T no SNC. Concomitantemente, há a expressão de (5) quimiocinas e (6) citocinas por linfócitos T encefalitogênicos e por células residentes ativadas do SNC, como os astrócitos e as micróglias, as quais em conjunto, recrutam um grande número de células mononucleares para o parênquima do SNC. Assim, a desmielinização dos tratos axonais ocorre (7) pela atividade fagocítica de células mononucleares ativadas e pela provável ação citotóxica, direta ou indireta, de moléculas efetoras solúveis como interferon γ (IFN-γ), linfotoxina α (LTα), fator de necrose tumoral α (TNF-α), óxido nítrico (NO), enzimas proteolíticas e radicais livres derivados do oxigênio, todos liberados por linfócitos CD4+ e macrófagos/micróglias ativados (Miller e Shevach, 1998).

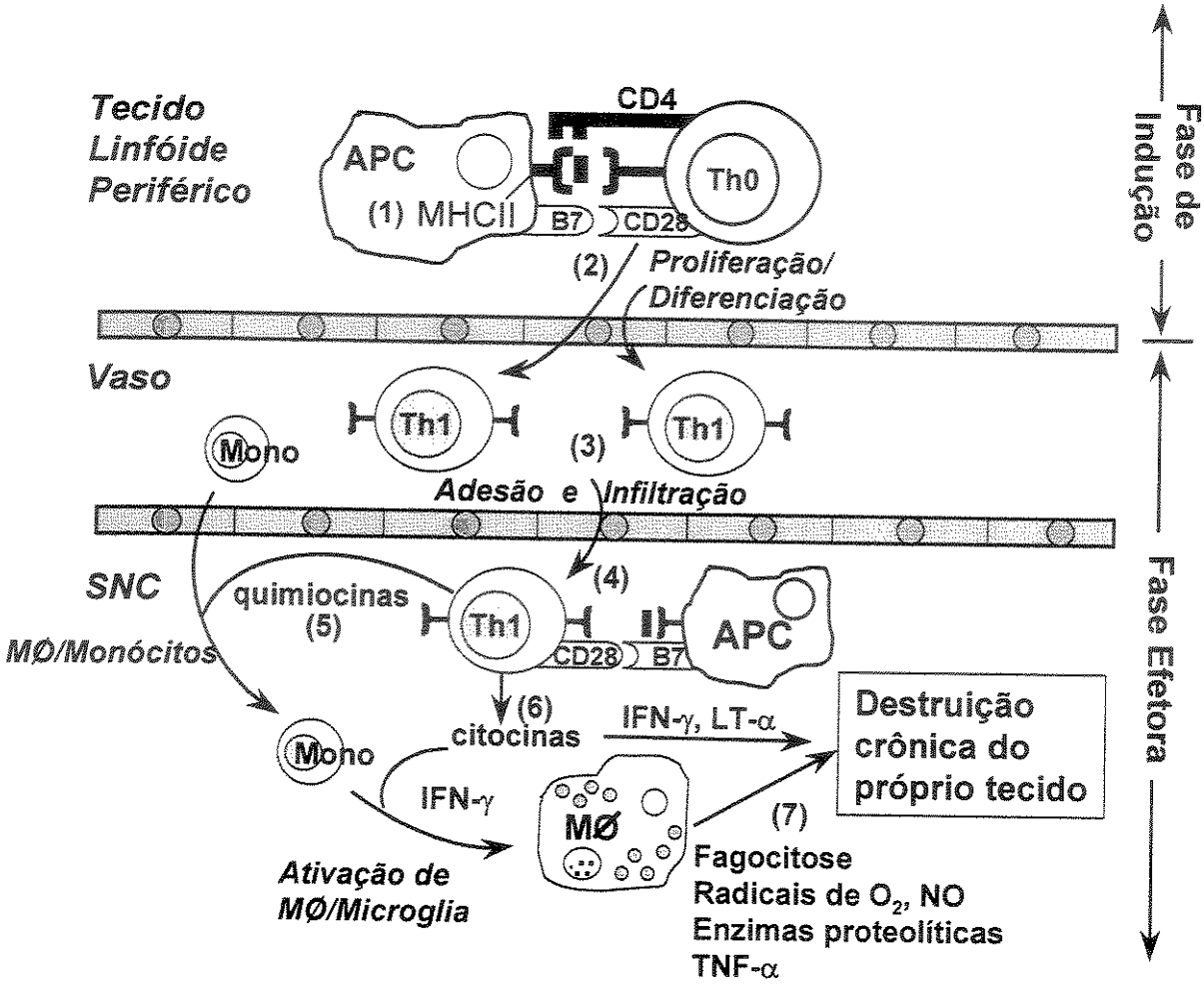
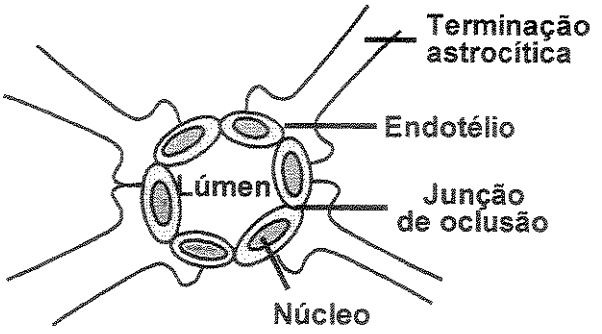


Figura 5. Modelo esquemático da patogênese e regulação da EAE (Miller e Shevach, 1998).

A barreira hemato-encefálica (BHE)

O SNC não possui tecido linfóide nem drenagem linfática definida, não sendo, portanto, exposto facilmente à estimulação antigênica. As células endoteliais do seu sistema vascular são unidas por junção de oclusão, formando uma barreira anatômica denominada BHE. Os astrócitos, cujas terminações ficam em contato com as paredes dos capilares, cobrindo até 70% de sua superfície, também contribuem para a formação da barreira (como mostrado na figura 6). É a BHE que regula a entrada de fluidos, eletrólitos, moléculas complexas e proteínas plasmáticas para o espaço extravascular, impedindo que moléculas de alto peso molecular e células tenham acesso livre ao SNC (Källén e Nilsson, 1986).



BHE

Figura 6. Esquema representativo da barreira hemato encefálica (Macedo et al., 1995).

Uma das principais características da EAE é o rompimento da BHE e o acúmulo de fluidos plasmáticos, levando ao surgimento de edema (Claudio et. al., 1995). Estudos realizados na EAE demonstram que linfócitos T ativados cruzam a BHE. Ao infiltrarem-se no parênquema cerebral, interagem com as células da glia e iniciam desta forma, a resposta inflamatória (Tran et al., 1998).

Os eventos que levam à quebra da BHE consistem na ativação de macrófagos e células T após a ligação com a ICAM-1, presente no endotélio cerebral, resultando na produção de moléculas inflamatórias como IFN-γ, IL-1 e NO que, posteriormente levam à alteração das propriedades da BHE (Merril e Murphy, 1997).

As proteínas da mielina

As principais proteínas da mielina são a proteína básica de mielina (MBP) e a proteína de proteolipídeo (PLP), mas há também a glicoproteína associada à mielina (MAG) e a glicoproteína de mielina do oligodendrócito (MOG; mostradas na figura 7).

É provável que a MBP, esteja associada à face citoplasmática da membrana, mediando a compactação entre duas superfícies citoplasmáticas adjacentes. A PLP é uma proteína integral de membrana, sendo sua suposta função, a de mediar a interação entre duas superfícies extracelulares opostas. A MAG, atua na

manutenção da estrutura periaxonal normal que, juntamente com a MOG, constituem os principais antígenos encefalitogênicos que desencadeiam os processos autoimunes na EAE (Beveniste, 1997; Ludiwin, 1997).

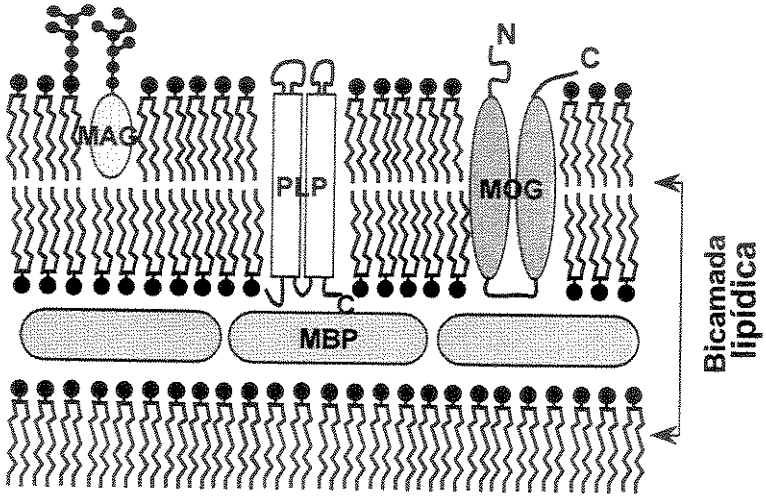


Figura 7. Esquema representativo das principais proteínas da mielina (modificado de Macedo et al., 1995). N=amino-terminal; C=carboxi-terminal.

Micróglias e macrófagos

As micróglias constituem um grupo de células da matéria branca e cinzenta do SNC (20% das células gliais). Sua morfologia é variável, mas em condições de repouso encontra-se ramificada e com o núcleo alongado.

Na EAE, durante a fase efetora, as micróglias são ativadas e sofrem mudanças morfológicas, assemelhando-se aos macrófagos, e mudanças funcionais que consistem no aumento da expressão dos antígenos do MHC e na liberação de substâncias mediadoras já mencionadas como moléculas citotóxicas, NO, intermediários reativos de oxigênio, proteases e citocinas (Gehrmann et al., 1995).

Os macrófagos e as micróglias são responsáveis pelos processos desmielinizantes característicos da EAE e da EM, devido à atividade fagocítica destas células (Bauer et al., 1994). O ataque do sistema imune é direcionado para os oligodendrócitos que são resposáveis pela formação e, juntamente com astrócitos, pela manutenção da mielina e assim atuando na manutenção do axônio (Ludwin, 1997).

Embora os macrófagos sejam células mediadoras da inflamação, Loughlin e col. (1994), verificaram que a presença de macrófagos em cultura de tecido cerebral induz um aumento dos níveis de MBP, PLP e MAG, sugerindo que desempenham importante função no processo de remielinização, por liberarem (pelo menos *in vitro*), fatores moduladores da proliferação, diferenciação e sobrevivência dos oligodendrócitos (Amor et al., 1997).

Astrócitos

Os astrócitos são células presentes tanto na matéria branca quanto na cinzenta do SNC. Desempenham a função de dar suporte estrutural para o tecido nervoso e atuam na transmissão sináptica (De Groot et al., 1991).

Na EAE e na EM, os astrócitos participam dos eventos imunológicos ocorridos no SNC. Podem ser induzidos a atuar de forma análoga aos macrófagos e células da micróglia. São capazes de responder ou secretar citocinas imunorregulatórias como a IL-1, IL-6, IL-8, IL-10, IL-12, fatores estimulantes de IFN-γ, TGF-β, TNF-α e quimiocinas (Beveniste, 1997).

Linfócitos T e citocinas

Linfócitos T CD4+ sob estímulo antigênico podem diferenciar-se nos sub-tipos Th1, Th2 ou Th3, dependendo do tipo de citocina secretada. As células Th1 estão envolvidas em reações de hipersensibilidade do tipo tardia , imunidade celular e viral, produzindo IL-2, linfotoxina, TNF-α e IFN-γ. As células Th2 estão envolvidas na proliferação e diferenciação de linfócitos B, participando na defesa contra parasitas e em processos alérgicos, produzindo IL-4, IL-5, IL-6, IL-10 e IL-13 (Olsson, 1995). Já as células Th3 consistem de uma linhagem distinta de linfócitos T, as quais secretam TGF-β (Hafler et al., 1997).

As citocinas têm função crucial no processo lesivo, atuando no início, propagação e regulação da lesão autoimune tecido-específica. As citocinas do tipo Th1 estão presentes nas lesões inflamatórias da EAE no SNC. Já as citocinas

expressas pelo tipo Th2 e Th3 estão associadas à fase de recuperação da EAE (Kuchroo e Weiner, 1998).

O estudo da EAE monofásica aguda em ratos Lewis, demonstrou que o aparecimento das citocinas é seqüencial. Inicialmente ocorre a produção de IL-12 pelas APCs, o que coincide com o aparecimento das primeiras células inflamatórias, antes do início dos sinais clínicos. Esta citocina promove a diferenciação das células Th1, que expressam IFN-γ, TNF-α e/ou LT-α. Posteriormente, na fase aguda da doença e paralelamente ao aumento da gravidade dos sinais clínicos, também são expressas IFN-γ e TNF-α. Estas citocinas irão desencadear a doença por lesão direta aos oligodendrócitos e à mielina, bem como pela ativação de macrófagos, que estão envolvidos na desmielinização. Durante a fase de recuperação da EAE ocorre o aumento da expressão das citocinas das células Th2, que secretam IL-4, IL-10 e IL-13 e atuam como desativadores de macrófagos. Além disso, há expressão do TGF-β, produzido por células Th2/Th3. A expressão de IL-4 não tem relação clara com a seqüência de eventos patológicos (Olsson, 1995; Navikas e Link, 1996).

1.2.1 A participação do NO

É bem estabelecido que o NO é capaz de modular a indução de uma resposta imune, a permeabilidade da BHE, difundindo-se de células para o SNC, e a resposta local no sítio inflamatório (Pender et al., 1997).

A primeira demonstração da participação do radical livre NO em EAE foi relatada por MacMicking e col. (1992). Eles mostraram o aumento da produção de intermediários reativos do nitrogênio bem como do oxigênio por células mononucleares e leucócitos polimorfonucleares periféricos, provenientes do SNC, e sistêmico de ratos com EAE.

Posteriormente, através da reação em cadeia da polimerase – transcriptase reversa (RT-PCR), outros autores detectaram a presença do mRNA para iNOS em tecido do SNC de ratos Lewis com EAE (Koprowski et al., 1993; Scott et al., 1996). Em seguida, o NO foi localizado na medula espinhal de camundongos, com EAE. Usando a técnica de EPR para detecção da formação do complexo Fe-NO, Lin e

col. (1993) observaram significante aumento dos sinais em todos os animais com EAE com relação aos controles, e que esse aumento era correlacionado com o curso clínico da doença. Concluíram então, que o NO é um importante mediador de uma doença crônica como a EM.

Hooper e col. (1995), usando um método modificado de "spin-trapping" para NO, *in vivo*, mostraram que, em ratos Lewis com EAE transferida de forma ativa por células T, a formação de grandes quantidades de NO eram provenientes da espinha dorsal 4-5 dias após a indução, correlacionada com a paralisia dos membros posteriores no 4° dia e paralisia geral no 5°. Também observaram que apesar da liberação de NO ser grande no cérebro, apresentava-se menor que na espinha dorsal, consistente com o curso ascendente da doença, com a espinha dorsal sendo o primeiro sítio de desenvolvimento da lesão.

Além desses achados, outros estudos têm mostrado que oligodendrócitos são mortos após exposição ao NO, sugerindo uma função tóxica para o NO na formação de lesões desmilienizantes na EAE (Merrill et al., 1993; Xiao et al., 1996). Contudo, o NO produzido por células fagocitárias ativadas pode exercer um efeito imuno-supressor pela inibição da proliferação de células T e infiltração de leucócitos (Kubes et al., 1991; Okuda et al., 1997).

Com isso, estudos voltados à eficácia da administração de inibidores da produção de NO no desenvolvimento da doença têm sido realizados. Os primeiros estudos terapêuticos foram realizados por Cross e col. (1994). Esses autores verificaram que a administração de aminoguanidina (AG, inibidor seletivo para a isoforma iNOS) era capaz de previnir os sinais clínicos causados pela EAE. O experimento foi realizado em camundongos SJL. Resultados similares foram obtidos por Brenner e col. (1997), e por Zhao e col. (1996) em ratos. Esses resultados indicaram que altos níveis de NO liberados pela enzima iNOS tinham atividade patogênica na EAE.

Zielasek e col. (1995) utilizando quatro inibidores de NOS (N-monometil-L-arginina, L-MMA; N-nitro-L-arginina, L-NA; aminoguanidina, AG; e N-nitro-L-arginina metil éster, L-NAME) em ambas formas de indução de EAE em ratos Lewis

(passiva, através da transferência de células T MBP-específicas, ou ativa, pela administração de MBP), verificaram que nenhum dos inibidores testados mostrou efeito benéfivo no curso clínico da doença. Já Zhao e col. (1996), verificaram que a AG usada no tratamento de EAE passiva causava proteção à doença e, Ruuls e col. (1996) verificaram que tratamento realizado com L-nitro arginina metil éster (L-NAME) ou L-nitro metil arginina (L-NMA) agravava a doença de forma ativa. Assim também foi observado por Gold e col (1997) utilizando o N-(1-iminoetil) L-lisina (NIL) e por Cowden e col. (1998) utilizando AG e L-NMA.

Conclui-se portanto que os resultados de tratamento da EAE com inibidores de NOS mostram-se totalmente divergentes, o que têm sido justificado por diversos fatores: forma de indução da doença, especificidade e doses dos inibidores, espécie e sexo do animal, período de administração, entre outros.

2. OBJETIVOS

Com base no relatado na Introdução sobre a participação do NO em doenças neurodegenerativas como a esclerose múltipla, os objetivos do presente trabalho foram:

1) Estudar a expressão e a atividade das isoformas de NOS dependentes e independente de Ca^{2+};

2) Estudar a influência dos cofatores necessários na biossíntese de NO,

3) Verificar a presença de fator(es) endógeno(s) inibidor(es) de NOS e,

4) Estudar a presença de proteínas contendo resíduos de nitrotirosina em homogenatos de cérebros de ratos Lewis durante o desenvolvimento do modelo de encefalomielite alérgica experimental.

3. MATERIAIS E MÉTODOS

3.1 Animais

Ratos Lewis (provenientes do Biotério Central da UNICAMP) de ambos os sexos, com peso corporal compreendido entre 200 - 250g foram utilizados para a indução da EAE. Os animais foram manipulados de acordo com as normas do Colégio Brasileiro de Experimentação Animal (www.fop.unicamp.br/ceea/etica_animal.htm).

3.2 Indução da EAE

A EAE foi induzida em ratos Lewis sob anestesia com halotano (Cristália Prod. Quim. Farm. Ltda, Brasil), através da inoculação de 100 μl de emulsão contendo proteína básica de mielina de cobaia (GP-MBP, 250 μg/ml, Sigma Chem. Co., EUA) e adjuvante completo de Freund (CFA, 3 mg/ml, Sigma Chem. Co., EUA) suplementado com *Mycobacterium tuberculosis* (Mt, 3mg/ml, Difco, EUA) em cada pata posterior, no coxim plantar (sub-cutâneo). Desta forma, cada animal recebeu a dose de 50μg da GP-MBP.

Os animais foram pesados e examinados diariamente. Os sintomas da doença foram determinados por estágios conforme segue: 0= sem sinais clínicos, I= perda do tônus da cauda, II= paralisia branda dos membros posteriores, III= paralisia severa dos membros posteriores, IV= tetraplegia e V= moribundos ou morte. No entanto, neste trabalho, utilizamos somente os animais que atingiram até o pico da doença (estágio III).

No final de cada estágio, um grupo de 3 a 5 animais foram anestesiados com halotano e amostras de sangue foram coletadas por exsangüinação. O plasma foi separado por centrifugação do sangue a 3000 rpm por 10 min e estocado a -20°C até serem processados. Os cérebros dos animais foram retirados e imediatamente congelados em nitrogênio líquido e estocados a -70°C para análises posteriores.

3.3 Medida da atividade de NOS

O método utilizado é o descrito por Förstermann e col.(1990) e está baseado na conversão da [^{3}H]L-arginina para [^{3}H]L-citrulina.

Os cérebros congelados a -70°C foram pesados e homogeneizados em homogeneizador ULTRA TURRAX T25 (JANKE & KUNKEL - IKA® Labortechnik, Alemanha) em 5 volumes de tampão de incubação (Tris-HCl 50 mM, pH 7.4) contendo 1 mM de PMSF e 1 mM de L-citrulina. Cinqüenta microlitros do homogenato foram incubados na presença de 1 mM de NADPH, 2 mM de $CaCl_2$ e 10 μM de L-arginina contendo 100,000 dpm de [2,3,4,5-^{3}H]L-arginina mono hidrocloreto (Amersham Life Science, Inglaterra) em um volume final de 100 μl a temperatura ambiente (25 - 27°C) durante 30 minutos, em duplicata. Todos os reagentes foram preparados em tampão de incubação (com exceção do PMSF e da L-citrulina). Após este período, a reação foi interrompida pela adição de 1 ml de tampão HEPES 20 mM, pH 5.4, contendo 1mM de EGTA e 1 mM de EDTA. Os tubos foram centrifugados (5' a 10.000 rpm em centrífuga Eppendorf Mod. 5412 - Alemanha) e os sobrenadantes aplicados em colunas contendo 0,6 ml de resina de troca iônica (tipo aniônica forte, Dowex AG 50X-8, Sigma Chem. Co., EUA). Os eluatos foram recolhidos em viais de cintilação. As colunas foram lavadas com 1 ml adicional de tampão HEPES e os eluatos foram combinados com aos anteriores. Após a adição de 10 ml de líquido de cintilação a radioatividade foi medida durante 1 min em espectrômetro de cintilação (BECKMAN - EUA).

Em cada ensaio foram realizados, em paralelo, controles farmacológicos da atividade enzimática que consistiram na omissão do $CaCl_2$ e na adição de 1 mM de EGTA no meio de incubação (afim de caracterizar o tipo de NOS quanto à dependência da presença de Ca2+ no meio de incubação), ou na adição de 1 mM de L-NAME (inibição específica para as NOS).

As contagens foram corrigidas por subtração do "branco" (onde o homogenato de tecido foi adicionado após o tampão HEPES) e da contagem obtida pelos tubos que obtinham a presença de L-NAME correspondente de cada amostra. Para o cálculo das atividades enzimáticas, as contagens (cpm) foram relacionadas à contagem total (os conteúdos destes tubos receberam [2,3,4,5-^{3}H]L-arginina mono hidrocloreto diretamente nos viais de cintilação) pela fórmula:

$$\textbf{pmol L-cit/min} = 1000 \times ((cpm_{amostra} - cpm_{branco}) - cpm_{L\text{-}NAME})/cpm\ totais/30$$

onde 1000 é a quantidade de L-arginina adicionada à mistura de incubação (em pmols) e 30 é o tempo de incubação (em min).

O conteúdo de proteínas foi determinado pelo método de Peterson (1977) e a atividade da NOS foi expressa como pmols de L-citrulina produzidos por minuto e por mg de proteína.

A atividade de NOS dependente de Ca^{2+} foi obtida pela subtração da atividade independente de Ca^{2+} (que possuia EGTA no meio de incubação) da atividade total.

Com a finalidade de caracterizar a atividade das isoformas de NOS (dependente ou independente de Ca^{2+}) presente nas MET dos animais controle ou com EAE quanto à dependência dos cofatores, outros experimentos foram realizados, medindo-se a atividade de NOS conforme Hiki e colaboradores (1992). Os cérebros foram homogeneizados em 5 volumes de tampão Tris 50 mM (pH 7,4) que continha 1 mM de L-citrulina, 0,1 mM de EDTA, 1 mM de ditiotreitol (DTT), 10 μg/ml de leupeptina, 10 μg/ml de inibidor de tripsina derivada de soja, 2 μg/ml de aprotinina e 1 mM de PMSF. Os homogenatos foram centrifugados a 10.000 *g* por 10 min e a atividade foi medida no sobrenadante. Cinquenta microlitros de amostra foram incubadas em um meio que continha 2 mM de $CaCl_2$, 1 mM de NADPH, 10 μM de L-arginina contendo 100,000 dpm de [2,3,4,5-^{3}H]L-arginina mono hidrocloreto e os cofatores CaM (10 μg/ml), FAD (10 μM), e BH_4 (100 μM) em um volume final de 100 μl a temperatura de 37°C, durante 15 minutos (em duplicata). A reação foi interrompida, o produto foi separado e o resultado foi expresso conforme descrito acima.

Aos efeitos de investigar a presença de possível(eis) substância(s) endógena(s) capaz(es) de afetar(em) a atividade de NOS dependente de Ca^{2+}, amostras (10 μl) de sobrenadante de homogenatos de cérebro total ou soros provenientes de animais controles ou com EAE (estágio III) foram acrescentadas ao meio de incubação (homogenatos de massa encefálica total provenientes de animais sem qualquer tipo de tratamento foram utilizados como fonte de NOS). Aos efeitos de caracterizar o efeito, tanto as amostras de homogenatos de cérebro total quanto os

soros foram desnaturados, através de aquecimento das amostras à 100°C (10 min para os homogenatos e 2 min para as amostras de soro).

3.4 Detecção das isoformas de NOS e de proteínas nitradas em resíduos de tirosina (NT) por Western blot

A expressão das diversas isoformas da NOS e de proteínas contendo resíduos nitrados de tirosina foi estudada através de técnica de Western blot.

Cem microlitros das amostras de homogenato de massa encefálica total (concentração de proteínas totais = 20 mg/ml) foram diluídas com 60 µl de tampão de Laemmli (0,0625 M de Tris-HCl, pH 6,8 contendo 2% de SDS, 10% de glicerol, 0,001% de azul de bromofenol e 5% de 2-mercaptoetanol) e fervidas durante 10 min.

Após rápida centrifugação a 10.000 *g* (30 seg), as proteínas das amostras foram separadas por eletroforese em gel de poliacrilamida (7%) contendo 0.1% de lauril sulfato de sódio (SDS - PAGE; Laemmli, 1970) para a detecção das isoformas da NOS. Para a detecção de NT, a concentração total de poliacrilamida no gel foi de 10%.

Os géis de concentração e de corrida foram preparados conforme descrito a seguir:

Gel de corrida:

Reagente	Gel 7% (ml)	Gel 10% (ml)
H_2O dest.	5,560	4,450
Glicerol 50%	0,095	0,095
TRIS 1,5M - pH 8,8	2,800	2,800
Acrilamida 30% + Bis-acrilamida 0,8%	2,600	3,700
SDS 10%	0,112	0,112
TEMED	0,010	0,010
Persulfato de amônio 10%	0,030	0,030

Gel de concentração:

Reagente	Gel 3% (ml)
H_2O dest.	3,150
TRIS 0,5M - pH 6,6	1,250
Acrilamida 30% + Bis-acrilamida 0,8%	0,500
SDS 10%	0,050
TEMED	0,006
Persulfato de amônio 10%	0,025

A composição do tampão utilizado na corrida eletroforética é a seguinte: TRIS (25 mM), glicina (192 mM) e SDS (0,1%) ajustado para pH 8,3.

A quantidade máxima de proteína aplicada em cada "lane" foi de 50 μg.

A separação eletroforética das proteínas foi realizada à intensidade de corrente constante (25 mA), durante aproximadamente 2 horas, resultando em valores de voltagem variando entre 100 a 180V. Posteriormente, as bandas protéicas foram transferidas eletroforeticamente através de sistema submerso para uma membrana de nitrocelulose, aplicando-se uma amperagem de 150-170 mA (voltagem ~50V) durante 2 horas. A composição do tampão empregado para a transferência eletroforética das proteínas para a membrana de nitrocelulose é a seguinte: TRIS (25 mM), glicina (192 mM), SDS (0,1%) e etanol (18%). Para comprovar a eficiência da transferência, os géis foram corados com corante Commassie blue (solução à 0,1% de Commassie brilliant blue em solução aquosa de ácido acético 5% contendo 25 % de etanol), e as membranas foram coradas com vermelho de Ponceau (solução à 2% de corante Ponceau em solução aquosa contendo 30% de ácido tricloro acético e 30% de ácido sulfosalicílico). Os sítios inespecíficos de ligação do anticorpo primário à membrana foram bloqueados mediante incubação da mesma com solução a 5% de leite em pó desnatado dissolvido em tampão TBS-t pH 7.4 (20 mM de TRIS-HCl, 8% de NaCl contendo 0,1% de Tween-20) sob agitação constante durante uma hora. A seguir, as membranas foram incubadas durante 15 - 18 horas, a 4°C com anticorpos primários específicos (diluídos em tampão TBS, sem a adição

de Tween-20) para as diferentes isoformas de NOS ou para resíduos de NT conforme indicado no quadro A:

Quadro A

Anticorpo	nNOS	eNOS	iNOS	NT
Hospedeiro	Coelho	Camundongo	Camundongo	Camundongo
Isotipo	Policlonal	IgG1	IgG2a	IgG
Conc. (μg/ml)	250	250	250	100
Diluição	1:1000	1:2500	1:2500	1:2000
Procedência	Transduction Lab., EUA	Transduction Lab., EUA	Transduction Lab., EUA	Upstate Biotech.,EUA

Após o término da incubação, as membranas foram lavadas (6 vezes durante 10 min) com tampão TBS-t e incubadas com anticorpos secundários conjugados com peroxidase de rabanete (HRP) ou fosfatase alcalina (AP), conforme mostrado no quadro B, durante 2 horas.

Quadro B

Anticorpo	nNOS	eNOS	iNOS	NT
Anticorpo secundário conjugado	Anti-coelho + HRP	Anti-camundongo + AP	Anti-camundongo + AP	Anti-camundongo + AP
Hospedeiro	Cabra	Coelho	Coelho	Coelho
Diluição	1:1000	1:1500	1:1500	1:3000
Kit de Revelação	4-cloro-1-naftol + H_2O_2	NBT/BCIP	NBT/BCIP	Quimio-Luminescência
Procedência	Bio Rad, EUA			

Em seguida, as membranas foram submetidas a uma nova série de lavagens com TBS-t e as bandas imunorreativas foram reveladas mediante um kit de revelação (quadro B).

O peso molecular das bandas foi calculado a partir das mobilidades relativas de proteínas marcadoras de peso molecular (faixa: 29 a 205 kDa, Sigma Chem. Co., EUA).

A intensidade das bandas (como medida do grau de expressão da proteína), foi determinada por análise densitométrica mediante o uso de software Sigma Gel (Sigma Chem. Co., EUA), e os valores foram expressos em unidades arbitrárias.

3.5 Nitração da albumina de soro bovino (BSA)

Aos efeitos de contarmos com um controle positivo apropriado de proteína nitrada em resíduo(s) de tirosina pelo peroxinitrito, procedemos à nitração da BSA. O procedimento envolve a reação de BSA com solução alcalina de peroxinitrito, de acordo com o relatado por Khan e col. (1998).

A solução de peroxinitrito foi preparada conforme descrito por Beckman e col. (1994b) pela reação de uma solução de nitrito de sódio ($NaNO_2$ 0,6M) com uma solução de peróxido de hidrogênio (H_2O_2 0,6M) acidificada com ácido clorídrico (HCl 0,7M) e rapidamente neutralizada com solução de hidróxido de sódio (NaOH 1,2M). O produto da reação foi colocado em tubos plásticos e congelados a -20°C. As gotículas oleosas formadas no topo dos tubos foram coletadas e diluídas em NaOH 1,2M. A concentração de peroxinitrito obtido foi determinado por absorbância a 302 nm utilizando-se um coeficiente de extinção molar de 1670 $M^{-1}cm^{-1}$.

A nitro-albumina foi preparada pela adição da solução alcalina de peroxinitrito (a concentração final de 1 mM) a uma solução de 2 mg/ml de BSA por 5 min e em temperatura ambiente. Uma alíquota de 50 µl foi separada, neutralizada com 25 µl de HCl 0,4M e devidamente diluída em tampão de Laemmli. Alíquotas equivalentes a 2,5 g de proteína nitrada (ou nativa, como controle negativo), foram submetidas a eletroforese e posterior análise por Western blot en todos os ensaios subsequentes de NT nas amostras de MET.

3.6 Determinação das concentrações séricas de NO_2^- e NO_3^-

Aos efeitos de evitar a ingestão de nitratos exógenos, os animais controles e animais com EAE no estágio III da doença foram deprivados de alimento sólido e receberam água destilada durante as 12 h prévias à coleta de sangue. Após este período, os animais foram anestesiados com halotano e amostras de sangue (3 ml) foram coletadas através da aorta abdominal descendente. Após retração do coágulo a temperatura ambiente, os tubos foram centrifugados (2000 *g* durante 15 min) e os soros foram separados e conservados a -20°C até serem analisados.

As concentrações de NO_2^-/NO_3^- foram determinadas por cromatografia líquida de alta eficiência (HPLC) de acordo com Muscará & de Nucci (1996). O sistema cromatográfico, permite a separação de ânions em coluna de troca iônica e detecção das espécies por posterior derivatização.

O sistema completo de HPLC consistiu de uma bomba (Apllied Biosystemms, EUA), uma coluna troca iônica (tipo catiônica forte; grupo funcional: amônio quaternário; tamanho de partícula: 5μ; Spherisorb SAX, 250 x 4,6 mm, Sigma-Aldrich, EUA) e de uma coluna recheada de Cd/Cu. A absorbância do efluente foi monitorada a 540 nm por um foto-detector de arranjo de diodos (modelo 1000 S, Apllied Biosystemms, EUA) e o sinal elétrico foi registrado em impressora.

A fase móvel utilizada para a separação dos ânions consistiu em uma solução de NH_4CL 0,06, pH 2,5 (fluxo 0,7 ml/min). Os padrões aquosos de KNO_3 e KNO_2 foram injetados diretamente no sistema (volume injetado: 400 μl). As amostras de soro foram diluídas 6 vezes com água e desproteinizadas por ultrafiltração através de tubos tipo Eppendorf com filtro de nitrocelulose com valor de corte de peso molecular 5 kDa (Sartorius ultrafiltration tubes, Alemânia) e 400 μl dos ultrafiltrados foram injetados no cromatógrafo.

3.7 Reação em cadeia da polimerase - transcrição reversa (RT-PCR)

Massa encefálica total de animais controle (n=3) e com EAE no estágio III (n=6) foram removidos, imediatamente congelados em nitrogênio líquido e mantidos a -70°C até o processamento das amostras.

Extração do RNA total:

O RNA total foi extraído usando o reagente Trizol (5 ml/g de amostra, GIBCO BRL, EUA). As amostras de MET foram homogeneizados em Trizol, e após 5 min, foi adicionado 1 ml de uma solução de clorofórmio / álcool isoamílico (1 parte de álcool isoamílico em 24 partes de clorofórmio) para cada 5 ml de Trizol. Após agitação vigorosa para a extração dos lipídeos, os tubos foram deixados 10 min em repouso e posteriormente centrifugados a 2000 *g*, (45 min a 4°C). A fase aquosa sobrenadante (600 µl) foi separada e o RNA precipitado pela adição de 500 µl de isopropanol. Após 10 min a temperatura ambiente, os tubos foram centrifugados (13.000 *g*, 10 min a 4°C). Os sobrenadantes foram descartados e o pellet de RNA foi lavado com 500 µl de etanol 75%. Após centrifugação (13000 *g*, 6 min a 4°C), os sobrenadantes foram desprezados e os tubos foram invertidos e apoiados sobre papel de filtro aos efeitos de eliminar restos de etanol.

Posteriormente, o RNA foi dissolvido com 50 µl de água tratada com DEPC. Alíquotas desta solução foram diluidas 1:1000, as absorbância00s medidas a 260 nm e as concentrações de RNA total foram calculadas considerando a relação: 1 AU = 40 µg/ml.

A integridade do RNA isolado foi verificada através de eletroforese em gel de agarose (0,8 % em TAE contendo 0,5 µg/ml de brometo de etídio, GIBCO BRL, EUA) e as bandas reveladas sob luz ultravioleta.

Transcrição do cDNA:

Cuidadosamente, 23 µl de H_2O (tratada com DEPC), 5 µl de oligo dT (GIBCO BRL, EUA) e 15 µg de RNA (amostra) foram misturados. A mistura foi aquecida 70°C durante 10 min e deixada posteriormente a temperatura ambiente durante 10 min.

Na seguinte ordem, foram adicionados: 10 µl de tampão de reação (5X first strand buffer), 2.5 µl de dNTP (10 mM, Amersham, Inglaterra), 5 µl de DTT (0,1 M) e 1 µl de inibidor de RNAses (RNA guard, Amersham, Inglaterra). A mistura foi

aquecida a 42°C durante 2 min, posteriormente adicionou-se 1 μl da enzima RT (transcriptase reversa – Superscript II, GIBCO BRL, EUA) e novamente aquecida a 42°C por 1 h.

Posteriormente, as amostras foram aquecidas a 70°C durante 15 min e a 37°C por 2 min. Adicionou-se, então, 1 μl de RNAse H (com a finalidade de se eliminar a RNAse do híbrido) e aqueceu-se a 37°C por durante 30 min. As amostras foram mantidas a -20°C até serem submetidas ao PCR.

PCR:

Os primers para as isoformas de NOS (segundo as seqüências utilizadas por Ferraz et al.,1997, para eNOS e iNOS, e por Swain et al., 1997 para nNOS) foram diluídos a uma concentração de 500 pmoles/μl (solução estoque) com H_2O Milli-Q autoclavada.

As seqüências dos primers são mostradas abaixo:

Nome	Seqüência do primer (5' → 3')
nNOS	CCCCGTCCTTTGAATACCAG
	CCGAGAGCCGAGGCCGAACA
iNOS	ACAACAGGAACCTACCAGCTCA
	GATGTTGTAGCGCTGTGTGTCA
eNOS	GGAGAAGATGCCAAGGCTGCTG
	CTTCCAGTGTCCAGACGCACCA
GAPDH	CGGAGTCAACGGATTTGGTCGTAT
	AGCCTTCTCCATGGTGGTGAAGAC

A reação de PCR foi realizada em um volume final de 50 μl que continha 5 μl de cDNA (amostra), 5 μl de tampão para PCR (10X), 1,5 μl de $MgCl_2$ (1,5 mM), 1 μl de dNTPs (0,2 mM), 0,5 μl do primer 1 (5 μM), 0,5 μl do primer 2 (5 μM), 0,3 μl do primer de GAPDH 5' (gliceraldeído 3-fosfato desidrogenase, controle interno, 3 μM),

0,3 µl do primer de GAPDH 3' (3 µM), 35,4 µl de H_2O para PCR e finalmente 0,5 µl de Taq polimerase (2 U, GIBCO BRL, EUA).

Os ciclos de amplificação foram realizados com desnaturação por 5 min a 94°C seguido por 33 ciclos de amplificação consistindo dos seguintes passos: denaturação a 94°C por 1 min, anelamento dos primers a 65°C por 45 seg e extensão a 72°C por 1,5 min. Após o último ciclo de amplificação, as amostras foram incubadas a 72°C por 7 min para extensão (Termociclador, PTC 100, MJ Research, EUA). Os tamanhos dos cDNAs resultantes dos primers para nNOS, iNOS e eNOS foram de 560 bp, 651 bp e 224 bp, respectivamente.

Separação dos produtos de PCR:

Alíquotas dos produtos da reação de PCR (aproximadamente 20 µl) previamente normalizadas para darem quantidades equivalentes do controle de GAPDH em todas as amostras foram submetidas a um gel de agarose 1,5% contendo 0.5 µg/ml de brometo de etídio. Os géis foram visualizados sob luz UV e as imagens foram capturadas usando um aparelho EagleEye (Amersham). A intensidade das bandas marcadas com brometo de etídio foram determinadas usando o FLA3000 Fluorescent Analyzer (FUJIFILM) para determinar o nível da expressão relativa de cada uma das isoformas.

3.8 Análise dos resultados

Os resultados experimentais foram expressos como média ± erro padrão da média e foram analisados pelo teste de ANOVA de um critério seguido do teste de Student - Newman - Keuls para múltiplas comparações. Valores de $p < 0,05$ foram considerados significantes.

4. RESULTADOS

A figura 8 mostra que na ausência dos cofatores exógenos (exceto NADPH), a atividade da NOS dependente de Ca^{2+} proveniente de homogenato de MET, foi significativamente diminuída em ratos com EAE no estágio III (5,9 ± 0,3) quando comparado com cada um dos grupos: controle (7,8 ± 0,7), EAE-0 (7,8 ± 0,3) ou EAE-I (7,8 ± 0,4 pmol L-citrulina/min/mg de proteína).

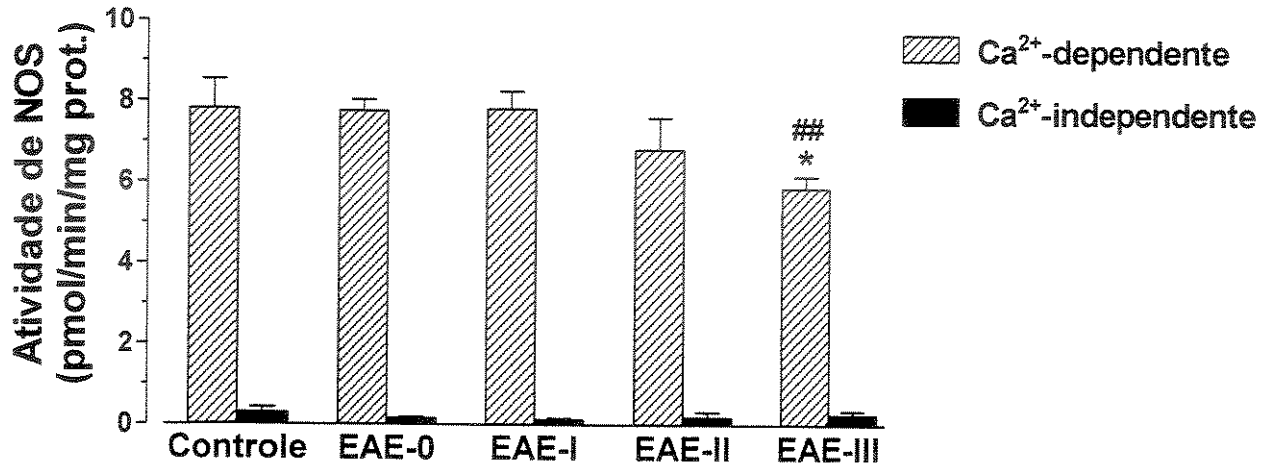


Figura 8. Atividade de NOS obtida de homogenato de MET de ratos controles e com EAE nos diferentes estágios. Nenhuma diferença significativa foi observada entre os grupos de NOS independente de Ca^{2+}.

*: $p<0,05$ vs Controle e EAE-I, e $^{\#\#}$:$p<0,01$ vs. EAE-0.

Contudo, e como mostrado na figura 9, nenhuma diferença significativa foi observada na atividade da NOS dependente de Ca^{2+} entre os grupos controle e EAE-III quando o ensaio foi realizado na presença de todos os cofatores testados: NADPH, FAD, CaM e BH_4 (81,0 ± 9,1 e 87,8 ± 7,2 pmol L-cit/min/mg proteína, respectivamente).

Nessas condições, a atividade da NOS independente de Ca^{2+} obtida de homogenatos de MET de animais com EAE-III mostrou-se maior do que aquela medida em animais controles (2,9 ± 0,5 e 1,9 ± 0,4 pmol L-cit/min/mg de proteína, respectivamente), embora esta diferença não seja estatisticamente significativa (Figura 9).

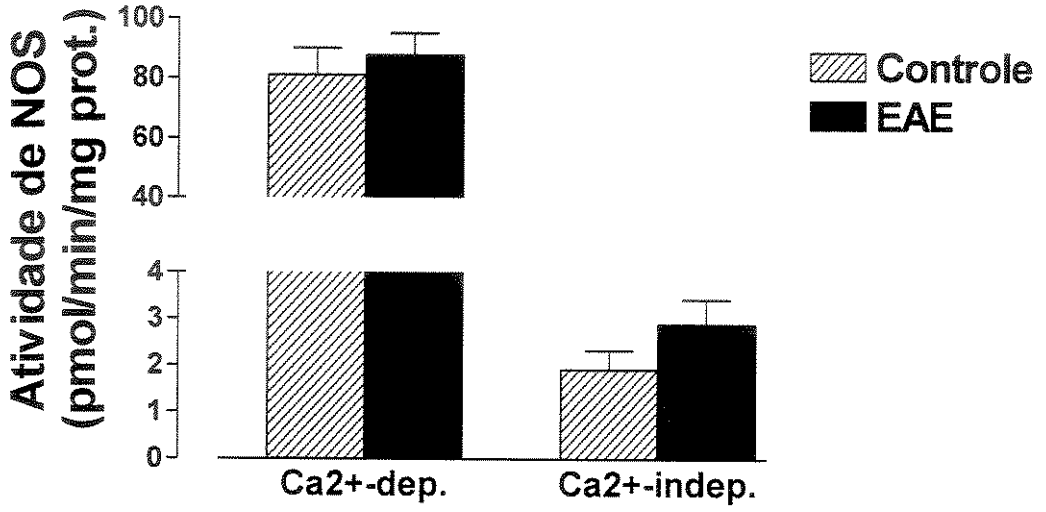


Figura 9. Atividade de NOS obtida de homogenatos de MET de ratos controles e com EAE no estágio III na presença dos cofatores NADPH, FAD, CaM and BH_4. Nenhuma diferença significativa foi observada entre os dois grupos nas atividades de NOS dependente ou independente de Ca^{2+}.

A figura 10 mostra que as atividades de NOS dependentes de Ca^{2+} medidas em homogenatos obtidos de MET de animais controle ou EAE-III foram igualmente inibidas (~30%) pela ausência de FAD no meio de incubação. Pelo contrário, a atividade de NOS independente de Ca^{2+} em homogenatos de animais com EAE-III não foi afetada pela ausência de qualquer um dos cofatores exógenos adicionados, enquanto que a omissão de FAD ou BH_4 do meio de incubação inibiu significativamente a atividade de NOS independente de Ca^{2+} dos animais controle (48,7 ± 13,0% e 62,7 ± 18,6%, respectivamente).

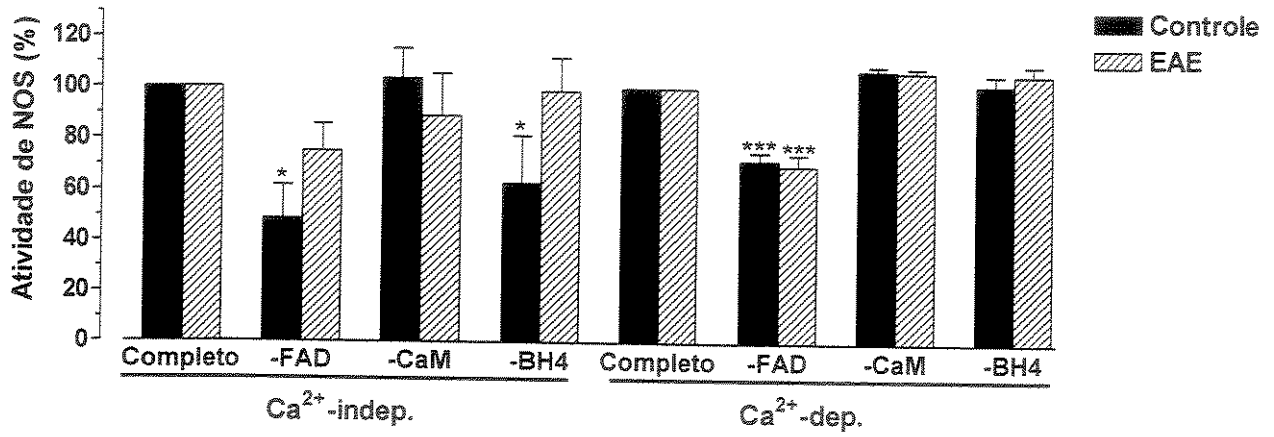


Figura 10. Efeito da omissão de cada um dos cofatores FAD, CaM e BH4 no meio de incubação sobre a atividade de NOS dependente e independente de Ca^{2+} de homogenatos de MET obtida de ratos controles e com EAE no estágio III.

*: $p<0,05$ e ***:$p<0,001$ vs. o respectivo grupo completo.

A figura 11 mostra que tanto os homogenatos de MET como os soros provenientes de animais controles inibiram a atividade da NOS dependente de Ca^{2+} (proveniente de homogenatos de MET de ratos normais) em grau maior do que aquele observado com os homogenatos de MET e soros de ratos com EAE-III (com homogenatos, controle: 20,1 ± 3,2, EAE III: 12,1 ± 2,1%, $p<0,05$; com soro, controle: 53,1 ± 1,8%, EAE-III: 38,7 ± 1,9%, $p<0,001$). Essas diferenças na inibição da atividade das NOS entre os grupos experimentais desapareceram quando ambos homogenatos e soros foram desnaturados por aquecimento (Figura 11).

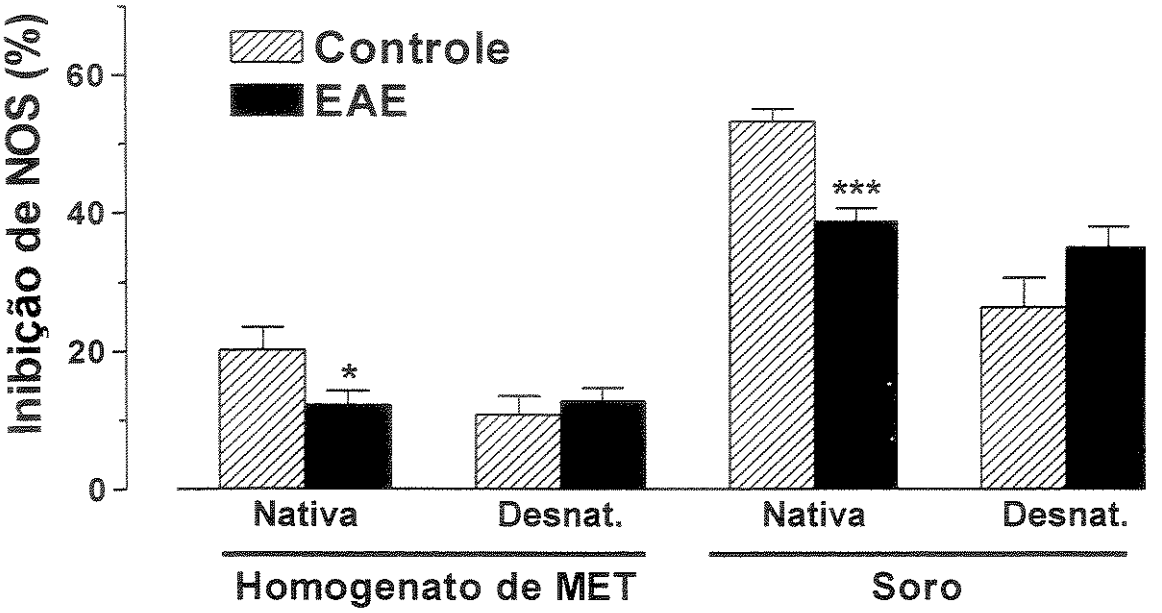


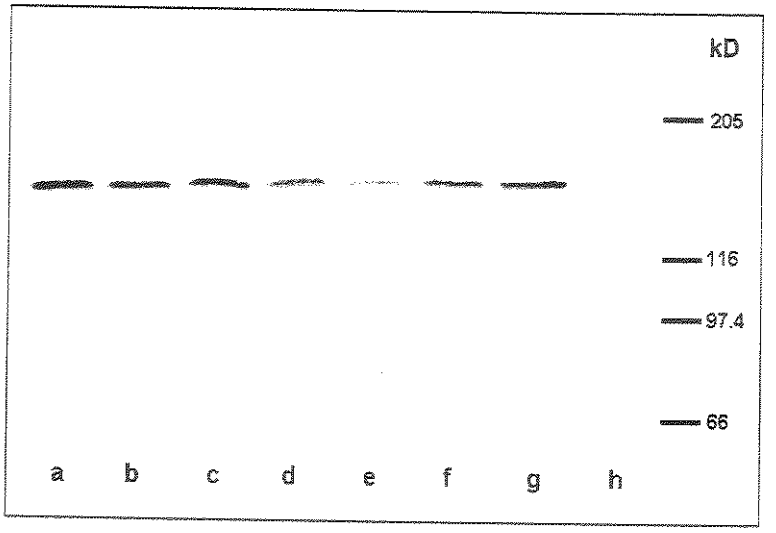
Figura 11. Efeito da presença de homogenato de MET e de soro obtidos de animais controle e com EAE no estágio III sobre a atividade de NOS de homogenato de massa encefálica total de animais normais.

*: $p<0,05$ e ***:$p<0,001$ vs. o respectivo Controle.

A análise da expressão das isoformas de NOS por Western blot monstrou que houve uma tendência ao aumento na expressão de nNOS em animais com EAE com o decorrer da doença quando comparado com o grupo de animais controle, como é mostrado na figura 12A e a análise densitométrica 12B.

No entanto, não foi possível detectar de forma precisa a expressão das isoformas iNOS e eNOS através dessa metodologia.

A)



B)

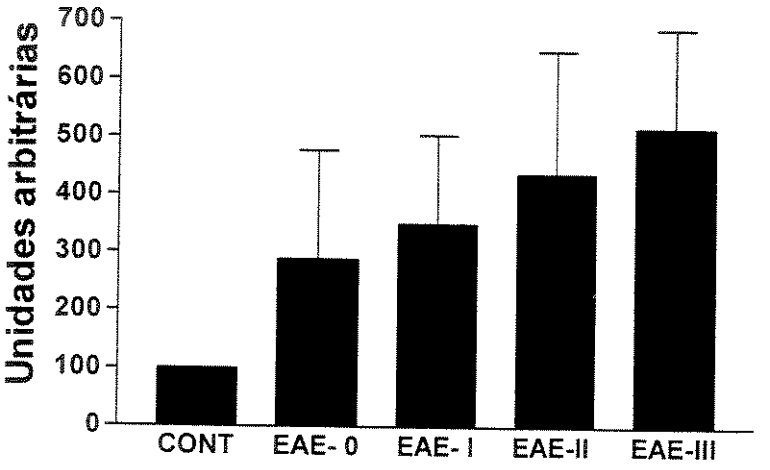
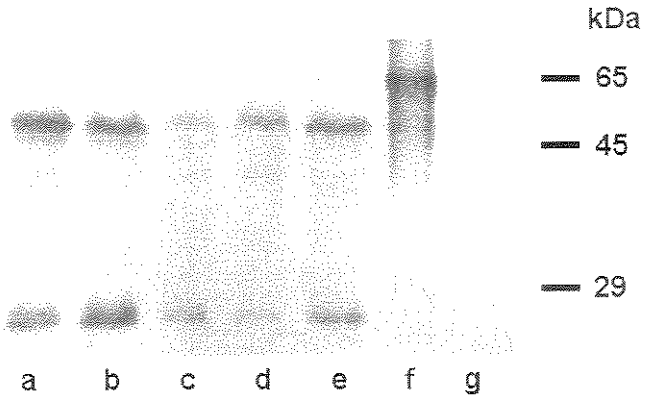


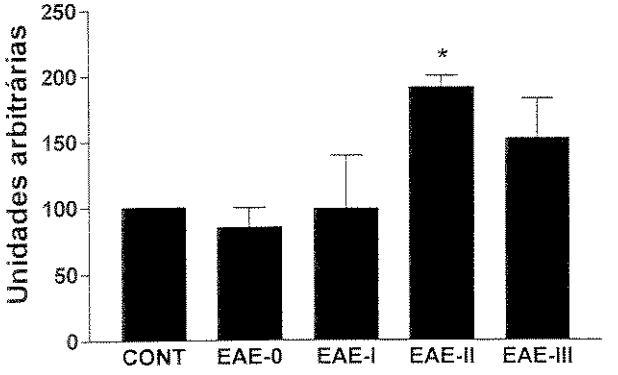
Figura 12. A) Western blot representativo (n = 3) de amostras de homogenatos de MET de ratos em diferentes estágios de EAE (25 µg) utilizando-se anticorpo policlonal anti-nNOS (dil. 1:1000). "Lanes" a, b: estágio III; c, d: estágio II; e, f: estágio I; g: estágio 0; h: controle. **B)** Intensidades relativas das bandas de nNOS (quantificadas por densitometria) para os diversos estágios de EAE em amostras de MET de ratos (vide Western blot do painel A). Não foi observada qualquer diferença significativa entre os diversos grupos.

A análise de Western blot para proteínas contendo NT em homogenatos de MET mostraram duas bandas principais (com peso molecular aproximado de 53 e 28 kD; figura 13A), e suas expressões foram significativamente aumentadas em ratos com EAE durante os estágios II e III quando comparado com o grupo controle (figuras 13B1 e 13B2).

A)



B1)



B2)

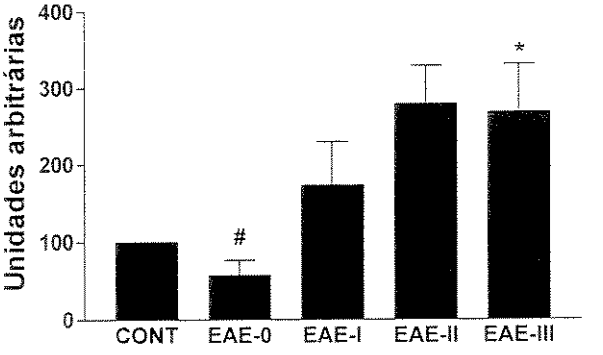


Figura 13. A) Western blot representativo (n = 4) de proteínas contendo resíduos de NT de amostras de homogenato de MET de rato em diferentes estágios de EAE (50 µg) utilizando-se anticorpo monoclonal anti-NT (dil. 1:2000). "Lane" a: estágio III; b: estágio II; c: estágio I; d: estágio 0; e: controle; f: nitro-albumina (2,5 µg); g: albumina nativa (2,5 µg).
B) Intensidades relativas das bandas de proteínas contendo resíduos de nitrotirosina (quantificadas por densitometria) para os diversos estágios de EAE em amostras de MET de ratos (vide Western blot do painel A). **B1**: banda correspondente a peso molecular 28 kD. **B2**: banda correspondente a peso molecular 51 kD. *: $p<0,05$ vs. grupo controle; #: $p<0,05$ vs. estágio II.

A concentração sérica de NO_3^- foi significativamente maior em ratos com EAE-III do que nos animais do grupo controle (104,6 ± 9,8 e 33,2 ± 6,75 μM, respectivamente; $p<0,01$), no entanto, nenhuma diferença significativa foi observada para os níveis circulantes de NO_2^- entre os dois grupos (Figure 14).

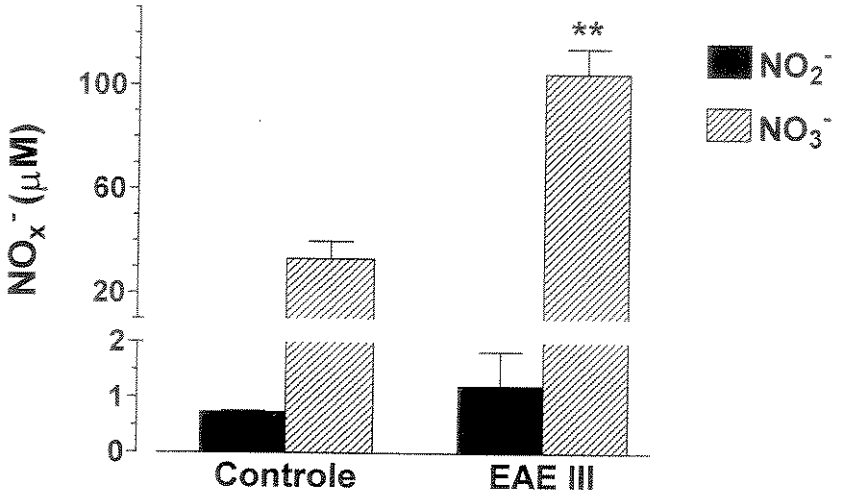


Figura 14. Concentrações séricas de nitrito (NO_2^-) e nitrato (NO_3^-) medidas em animais controle e EAE-III. **: $p<0,01$ vs. grupo controle para NO_3^-. Não foram observadas diferenças significativas entre os grupos para NO_2^-.

A análise de RT-PCR revelou um aumento nos níveis de mRNA para iNOS nos homogenatos de MET obtidos de ratos com EAE-III em comparação com os provenientes dos animais controle (Figura 15). Por outro lado, nenhuma alteração significativa foi observada nos níveis de mRNA para nNOS e eNOS.

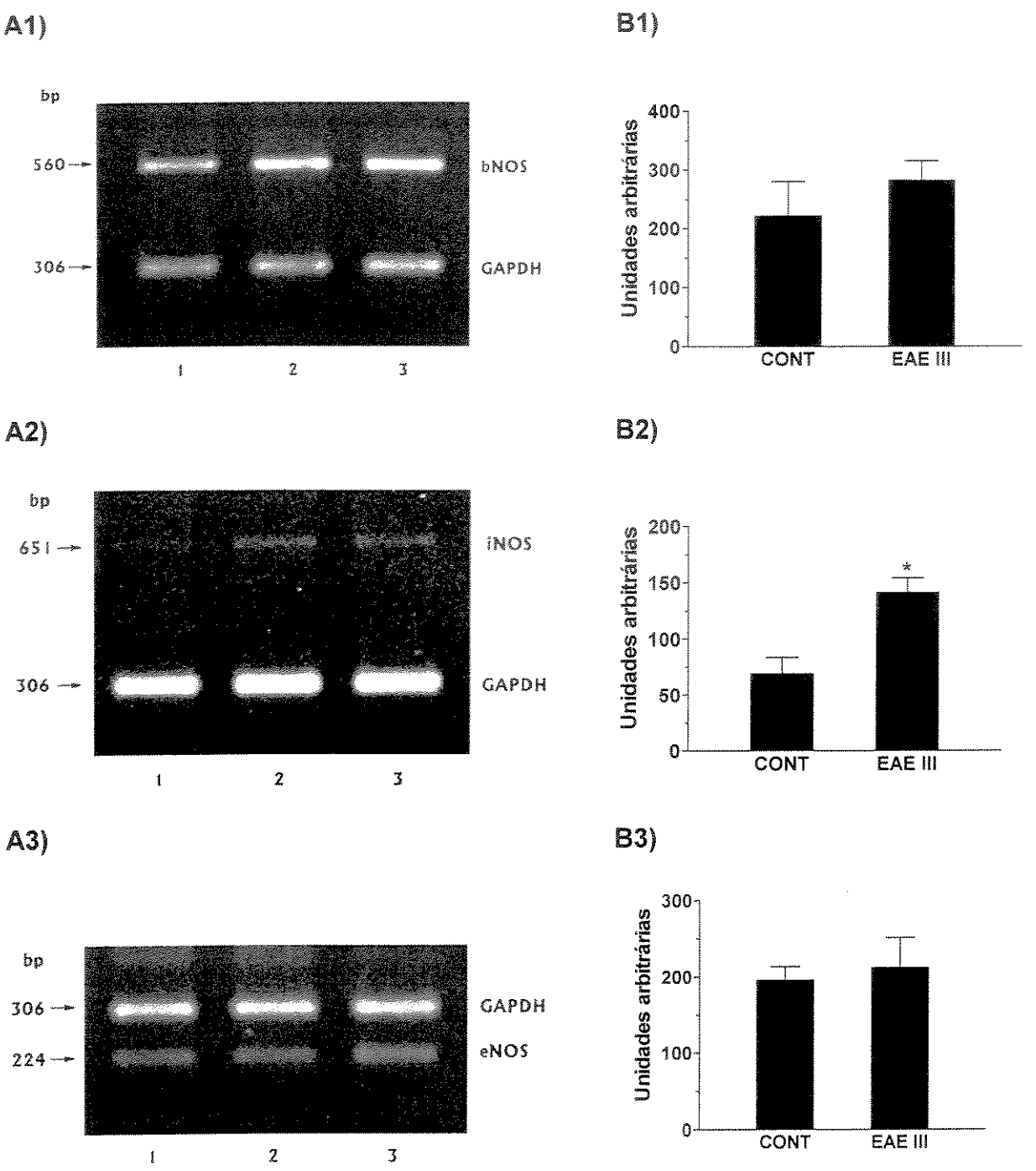


Figura 15.- A) Análise de mRNA por RT-PCR: A1) nNOS; A2) iNOS e A3) eNOS em amostras de homogenatos de MET obtidas de animais controle ou com EAE no estágio III. GAPDH foi empregado como controle interno. "Lane" *1*: Controle, "Lanes" *2* e *3*: EAE-III. **B)** Níveis de expressão de mRNA para nNOS, iNOS e eNOS (B1, B2 e B3, respectivamente) estimados por densitometria das bandas mostradas nos painéis A.
*: $p<0,001$ vs. o grupo controle.

5. DISCUSSÃO

Os resultados expostos mostram que na ausência de qualquer cofator de NOS adicionado exogenamente (com exceção feita para o NADPH), a atividade da NOS dependente de Ca^{2+} presente em homogenatos de MET de ratos com EAE diminuiu de forma paralela à severidade da doença, assim como em relação à atividade observada nos homogenatos de MET provenientes de animais controle. Nessas condições, nenhuma diferença significativa foi observada entre os grupos para a atividade independente de Ca^{2+}. Também, em termos de expressão de nNOS e eNOS, nenhuma diferença significativa foi observada entre os grupos, quando analisadas por RT-PCR, o que levou-nos então, a investigar a possível presença de fator(es) inibitório(s) que poderia(m) estar afetando a atividade da enzima dependente de Ca^{2+} e/ou alguma possível deficiência de cofatores de NOS nos animais com EAE.

Na presença de todos os cofatores essenciais de NOS (NADPH, FAD, CaM e BH_4), nós observamos que a atividade da NOS dependente de Ca^{2+} presente em homogenatos de MET de ratos com EAE, era restaurada aos níveis obtidos dos animais do grupo controle, e que a atividade da NOS independente de Ca^{2+} era ainda semelhante entre ambos os grupos, apesar da tendência a aumentar no grupo de animais com EAE, assim como um significativo aumento de mRNA para iNOS (Figura 15).

Estudamos então, a atividade da NOS dependente e independente de Ca^{2+} na ausência de cada um dos cofatores adicionados anteriormente. A omissão de FAD no meio de incubação resultou em uma diminuição da atividade da NOS dependente de Ca^{2+} proveniente de homogenatos de MET de ambos os grupos. Uma queda significativa foi observada na atividade da NOS independente de Ca^{2+}, decorrente da ausência de FAD ou BH_4 no meio de incubação nos homogenatos de MET provenientes dos animais do grupo controle. Por outro lado, nenhuma alteração significativa foi observada na atividade dessa isoforma nas amostras provenientes de animais doentes na omissão de qualquer um dos cofatores adicionados.

Tanto o FAD como o BH_4 são cofatores essenciais para a atividade funcional das NOS pois estão envolvidos na transferência de elétrons nos dois passos da

biossíntese do NO (Marletta, 1988; Marletta, 1993; Masters, 1994; Knowles e Moncada, 1994; Stuher, 1997).

Nesse ponto, é muito importante mencionar que resultados prévios mostram que citocinas pró-inflamatórias estimulam a produção de BH_4 em células endoteliais (Katusic et al., 1998) bem como em células do músculo liso vascular (Hattori et al., 1998). Walter e col. (1998) mostraram que o TNF-α é o mais potente estímulo para a síntese *de novo* de BH_4.

Okuda e col. (1995) mostraram que os níveis de mRNA e a expressão protéica de iNOS e de algumas citocinas pró-inflamatórias (IL-1α, IL-1β, IL-2, IL-6, IFN-γ, TNF-α e TNF-β) medidas no líquido cefalorraquidiano (líquor) de ratos com EAE foram positivamente correlacionados com o grau de severidade da doença. Também demonstraram que não ocorre alteração nos níveis das citocinas imunorregulatórias (IL-4, IL-10 e TGF-β).

Em estudos com pacientes com EM, Johnson e col. (1995) além de encontrarem altos níveis de nitrato e nitrito no líquor desses pacientes, também encontraram um aumento nos níveis de neopterina, precursor da BH_4 quando comparados com indivíduos normais.

Considerando-se esses resultados e que a produção de citocinas pró-inflamatórias encontra-se aumentada em animais com EAE no pico de severidade da doença, nós podemos supor que altos níveis de BH_4 endógeno em ratos com EAE são responsáveis pela falta de efeito observado na atividade de iNOS quando o BH_4 exógeno não foi adicionado ao meio de incubação (Figura 10).

Por outro lado, uma elevada atividade da glutationa redutase (um índice empregado clinicamente para estimar os níveis de riboflavina presentes no organismo) tem sido relatada em líquor de pacientes com EM (Calabrese et al., 1994). De forma semelhante às observações feitas acima com relação a BH_4, esses achados clínicos poderiam explicar o motivo pelo qual a omissão de FAD exógeno do meio de incubação não teve efeitos significantes na atividade de NOS independente de Ca^{2+} presente no cérebro de ratos com EAE, mas que em contrapartida, inibiu em torno de 50% a atividade da enzima presente em cérebro de

ratos controles (Figura 11). Contudo, nós não tivemos uma explicação satisfatória para os efeitos da omissão desse cofator na atividade de NOS dependente de Ca^{2+}, onde decréscimos semelhantes foram observados em ambos os grupos de animais.

Apesar da inabilidade de detectarmos a atividade da NOS independente de Ca^{2+} em homogenatos de MET de animais com EAE (estágio III), altos níveis de nitrato circulante, aumento de mRNA para iNOS e proteínas nitradas em resíduos de tirosina foram encontradas nesse grupo de animais, concluindo-se uma exacerbada produção de NO em comparação com o grupo controle. Esta conclusão, é reforçada por relatos prévios que mostram altos níveis de produtos finais de NO em amostras de soro de rato com EAE (Cross et al., 1997; Cowden et al., 1998; O'Brien et al.,1999) e em amostras de líquor coletadas de pacientes com EM (Yamashita, 1997; Giovannoni, 1998; Giovannoni et al., 1998).

Como mostrado na Figura 11, tanto homogenatos de MET como soro provenientes de animais com EAE inibiram a atividade de NOS dependente de Ca^{2+} em menor grau do que o observado com amostras obtidas de animais controles. No entanto, essas diferenças entre os dois grupos desapareceram quando as amostras de homogenatos de MET ou soro foram desnaturadas por aquecimento. Interessantemente, esse processo de desnaturação foi efetivo na diminuição da atividade de inibição para os níveis presentes em amostras de animais controles, sugerindo assim, que níveis endógenos de inibidores de NOS presentes em ratos controles encontram-se pelo menos diminuídos em ratos com EAE. Esses resultados são, em princípio, contraditórios, considerando que a diminuição significativa na atividade de NOS dependente de Ca^{2+}, observada em ratos com EAE, não pode ser explicada em termos de deficiência de cofatores da enzima. Dados de literatura descrevem que resíduos de arginina em proteínas são metiladas por uma família de proteínas nomeadas de N-metiltransferases (PRMTs; Clarke, 1993). Essas enzimas catalisam a metilação do nitrogênio do grupo guanidino da arginina para produzirem N^G-monometil-L-arginina (L-NMMA), N^G,N^G-dimetil-L-arginina (dimetil-arginina assimétrica; ADMA) e N^G,N'^G-dimetil-L-arginina (dimetil-arginina simétrica; SDMA). Quando proteínas contendo esses resíduos sofrem

proteólise ocorre a liberação dessas metil-argininas livres (Kakimoto e Akazawa, 1970). As metil-argininas ocorrem endogenamente e elas têm sido detectadas em células imunes (Kimoto et al., 1993), em neurônios (Ueno et al., 1992) e em plasma humano (Vallence et al., 1992). L-NMMA e ADMA (mas não SDMA) são inibidores não específicos das três isoformas de NOS e podem alterar a atividade da NOS (Vallence et al., 1992). Elevadas concentrações de ADMA têm sido detectadas em plasma de pacientes ou animais experimentais com hiperlipidemia (Yu et al., 1994), doenças renais (Vallence et al., 1992) ou com arteriosclerose (MacAllister et al., 1994) bem como em pacientes com esquizofrenia (Das et al., 1996).

Rawal e colaboradores (1995) demonstraram que os valores da excreção urinária de ADMA e SDMA provenientes de pacientes com esclerose múltipla foi 20% menor daqueles obtidos dos indivíduos controles. De acordo com essa última observação podemos, pelo menos em parte, justificar nossos achados, mas uma interpretação fisiológica conclusiva fica ainda por ser estabelecida.

O NO pode se combinar com o ânion superóxido para dar origem ao peroxinitrito que é um forte oxidante e agente nitrante que pode promover peroxidação lipídica, nitração de proteínas em resíduos de tirosina e ainda gerar radical hidroxila que é altamente tóxico (Beckman et al., 1990). Dessa forma, o papel do NO no SNC tem sido extensivamente estudado nesse modelo de esclerose múltipla experimental, embora, os resultados ainda não sejam conclusivos.

Foi Bagasra e col. (1996) que além de demonstrarem a expressão de mRNA para iNOS em todos os cérebros de pacientes com EM examinados e não nos cérebros normais, mostraram também a presença de proteínas nitradas em lesões da EM com anticorpo anti-nitrotirosina, co-localizando-se com o mRNA da iNOS e proteínas nessas células (macrófagos e microglia).

O NO pode causar danos em regiões específicas de uma maneira dose-, tempo- e sítio-dependente mas pode também contribuir significativamente, e talvez mais importantemente, para uma atenuação do estado da doença (para revisão, vide Willenborg et al., 1999). Pasquet e col. (1996) demonstraram que o peroxinitrito pode atuar como um potente inibidor da síntese de NO, e que essa inibição seria

preferencialmente devida à oxidação de grupos sulfidrilas do que à nitração de resíduos de tirosina da enzima.

Por outro lado, vários trabalhos já demonstraram que o NO pode causar inibição de sua própria síntese (Rogers e Ignarro, 1992; Buga et al., 1993; Rengasamy e Johns, 1993; Griscavage et al., 1993; Assreuy et al., 1993).

Estudos farmacológicos utilizando os inibidores de NOS também levam a conclusões contraditórias sobre o papel do NO no estado clínico de animais com EAE. Os primeiros estudos terapêuticos sugerindo uma função do NO na EAE foram realizados por Cross e col. (1994) e seus resultados, foram mais tarde, confirmados por Brenner e col. (1997). Esses autores, verificaram que a administração de aminoguanidina (AG, inibidor específico para iNOS) era capaz de previnir os sinais clínicos causados pela EAE em camundongos SJL. Resultados semelhantes foram obtidos em ratos por Zhao e col (1996). Esses resultados indicaram que altos níveis de NO produzido pela iNOS seriam fator patogênico no processo da EAE.

Por outro lado, Zielasek e col. (1995) demonstraram que a administração de diferentes inibidores de NOS não tiveram nenhum efeito terapêutico significante sobre a EAE em ratos. Ainda, esses autores mostraram que a AG agravou "levemente" o estado clínico dos animais com EAE; observações que foram mais tarde confirmadas por Cowden e col. (1998) empregando ambos AG e L-NMMA. Reforçando essas conclusões, outros autores também mostraram que a administração de inibidores de NOS não-seletivos, tais como L-nitro arginina metil éster (L-NAME) e L-nitro metil arginina (L-NMMA, Ruuls e col, 1996), ou altamente seletivo para a isoforma iNOS como o N-(1-iminoetil) L-lisina (L-NIL, Gold e col, 1997), tiveram efeitos clínicos negativos, sugerindo que os efeitos benéficos de NO poderiam ser explicados com base em sua atividade imuno-supressora.

Portanto, a função do NO na EAE, bem como na EM, ainda não é bem estabelecida. Dessa forma, os efeitos causados pela administração de qualquer inibidor de NO em animais com EAE devem ser analisados com muita cautela, uma vez que poderão ser futuramente utilizados no uso terapêutico em clínicas para a prevenção da EM humana.

6. CONCLUSÃO

Com base nos resultados apresentados, nós concluímos que ocorre aumento da expressão da iNOS durante estágios mais avançados da EAE, o qual seria responsável pelo aumento da produção de NO sistêmico e ao aumento dos níveis de proteínas nitradas no SNC. Em paralelo a esses resultados, uma diminuição da atividade de NOS constitutiva ocorre provavelmente devido a inibição pelo excesso de NO/peroxinitrito formado, mas não devido a presença de inibidores endógenos ou a qualquer deficiência de cofatores.

Por outro lado, se a atividade *ex-vivo* da NOS independente de Ca^{2+} presente no cérebro de ratos com EAE não é afetada pela omissão de ambos os cofatores FAD e BH_4 e se a presença de um inibidor circulante de NOS (termolábil) não foi observada nesses animais, isso tudo poderia justificar um mecanismo fisiológico tendendo a potencializar a atividade imuno-supressora do NO derivado da iNOS. Contudo, experimentos adicionais devem ser realizados com a finalidade de confirmar essa hipótese.

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Effect of *Tityus serrulatus* scorpion venom on the rabbit isolated corpus cavernosum and the involvement of NANC nitrergic nerve fibres

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1 The effect of *Tityus serrulatus* scorpion venom and its toxin components on the rabbit isolated corpus cavernosum was investigated by use of a bioassay cascade.

2 *Tityus serrulatus* venom (3–100 μg), acetylcholine (ACh; 0.3–30 nmol) and glyceryl trinitrate (GTN; 0.5–10 nmol) dose-dependently relaxed rabbit isolated corpus cavernosum preparations precontracted with noradrenaline (3 μM). The selective soluble guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ; 30 μM) increased the basal tone of the rabbit isolated corpus cavernosum and abolished the relaxations induced by the agents mentioned above. Methylene blue (30 μM) also inhibited the relaxations induced by *Tityus serrulatus* venom but, in contrast to ODQ, the inhibition was irreversible.

3 The non-selective NO synthase (NOS) inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10 μM) and N^{G}-iminoethyl-L-ornithine (L-NIO; 30 μM) also increased the tone of the rabbit isolated corpus cavernosum and markedly reduced both ACh- and *Tityus serrulatus* venom-induced relaxations without affecting those evoked by GTN. The inhibitory effect was reversed by infusion of L-arginine (300 μM), but not D-arginine (300 μM). The neuronal NOS inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM, 100 μM) did not affect either the tone of the rabbit isolated corpus cavernosum or the relaxations induced by ACh, bradykinin (Bk), *Tityus serrulatus* venom and GTN. TRIM was approximately 1,000 times less potent than L-NAME in inhibiting rabbit cerebellar NOS *in vitro*, as measured by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline.

4 The protease inhibitor aprotinin (Trasylol; 10 μg ml^{-1}) and the bradykinin B_2 receptor antagonist Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7, Oic^8]-BK; 50 nM) did not affect the rabbit isolated corpus cavernosum relaxations induced by *Tityus serrulatus* venom. The ATP-dependent K^+ channel antagonist glibenclamide (10 μM) and the Ca^{2+}-activated K^+ channel antagonists apamin (0.1 μM) and charybdotoxin (0.1 μM) also failed to affect the venom-induced relaxations. Similarly, the K^+ channel blocker tetraethylammonium (TEA; 10 μM) had no effect on the venom-induced relaxations.

5 Capsaicin (3 and 10 nmol) relaxed the rabbit isolated corpus cavernosum in a dose-dependent and non-tachyphylactic manner. Ruthenium red (30 μM), an inhibitor of capsaicin-induced responses, markedly reduced the relaxations caused by capsaicin, but failed to affect those induced by *Tityus serrulatus* venom. L-NAME (10 μM) had no effect on the capsaicin-induced relaxations of the rabbit isolated corpus cavernosum.

6 The sodium channel blocker tetrodotoxin (TTX; 1 μM) abolished the relaxations of the rabbit isolated corpus cavernosum induced by *Tityus serrulatus* venom without affecting those evoked by capsaicin, ACh and GTN. Tetrodotoxin (1 μM) also promptly reversed the response to the venom when infused during the relaxation phase.

7 The bioassay cascade of the toxin components purified from *Tityus serrulatus* venom revealed that only fractions X, XI and XII caused dose-dependent relaxations of the rabbit isolated corpus cavernosum and these were markedly reduced by either TTX (1 μM) or L-NAME (10 μM).

8 Our results indicate that *Tityus serrulatus* scorpion venom (and the active fractions X, XI and XII) relaxes rabbit corpus cavernosum via the release of NO. This release is specifically triggered by the activation of capsaicin-insensitive cavernosal non-adrenergic non-cholinergic (NANC) fibres, that may possibly be nitrergic neurones. *Tityus serrulatus* venom may therefore provide an important tool for understanding further the mechanism of NANC nitrergic nerve activation.

Keywords: Scorpion venom; nitrergic nerves; tetrodotoxin; non-adrenergic non-cholinergic nerves; nitric oxide; ODQ

Introduction

Scorpion toxins have been extensively used to study the activation of both Na^+ (Barhanin *et al.*, 1982; Nagy, 1988; Yatani *et al.*, 1988; Kirsch *et al.*, 1989) and K^+ (Blaustein *et al.*, 1991; Rogowski *et al.*, 1994; Vatanpour & Harvey, 1995) channels. *Tityus serrulatus* is the most dangerous scorpion of the subfamily Tityinae because of the high toxicity of its venom and its widespread distribution in populous urban centres of southeastern Brazil (Bucherl & Diniz, 1978). The most important symptoms of the human envenomation by *Tityus*

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serrulatus are intense local pain and an immediate local burning sensation which may last from a few minutes to several hours. These symptoms may be accompanied by autonomic dysfunctions (mydriasis, salivation, sphincter relaxation) and cardiovascular disorders characterized by short-lived hypotension followed by a prolonged increase in blood pressure, arrhythmia and bradycardia (Corrado *et al.*, 1974). *Tityus serrulatus* venom is known to act on nerve endings to stimulate the release of either acetylcholine (Gomez *et al.*, 1973; Oliveira *et al.*, 1989; Vatanpour & Harvey, 1995) or catecholamines (Corrado *et al.*, 1974; Moss *et al.*, 1974; Langer *et al.*, 1975) from different organs and tissues. Interestingly, the venom of the African scorpion *Leiurus quinquestriatus quinquestriatus* relaxes the rat isolated anococcygeus muscle via nitric oxide (NO) release, possibly due to persistent depolarization of peripheral non-adrenergic non-cholinergic (NANC) nerves (Gwee *et al.*, 1995). Since NANC nerve stimulation causes corpus cavernosum relaxation (Ignarro *et al.*, 1990), we have investigated the effects of *Tityus serrulatus* scorpion venom on the rabbit isolated corpus cavernosum.

Methods

Rabbit corpus cavernosum preparation

Male New Zealand white rabbits (1.5–2.5 kg, provided by CEMIB-UNICAMP) were anaesthetized with pentobarbitone sodium (Sagatal, 30–40 mg/kg^{-1}, i.v.) and exsanguinated via the carotid artery. Following penectomy, the rabbit corpus cavernosum was dissected in the Krebs solution and cleared of the tunica albuginea and surrounding tissues. Strips of rabbit isolated corpus cavernosum were superfused in a cascade system (Vane, 1964) with warmed (37°C) and oxygenated (95%O_2 + 5%CO_2) Krebs solution at a flow rate of 5 ml min^{-1}. The tissue responses (tension of 2.5 g) were detected with auxotonic levers attached to Harvard heart/smooth muscle isotonic transducers and displayed on a Watanabe multichannel pen recorder (model WTR 381). After a 60–90 min period of equilibration, rabbit isolated corpus cavernosum strips were precontracted with noradrenaline (3 μM) in order to increase the basal tone. The tissues were continuously infused with indomethacin (5.6 μM) to inhibit the generation of cyclo-oxygenase products.

Tityus serrulatus venom and other substances (glyceryl trinitrate, acetylcholine, bradykinin, substance P, vasoactive intestinal peptide, calcitonin gene-related peptide, cromakalim and capsaicin) were administered as single bolus injections (10–50 μl). N^{ω}-nitro-L-arginine methyl ester (L-NAME), D-NAME, L-arginine, D-arginine, N^{G}-iminoethyl-L-ornithine (L-NIO), 1-(2-trifluoromethylphenyl) imidazole (TRIM), 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ), aprotinin (Trasylol), Hoe 140 (D-Arg-[Hyp^3,Thi^5,DTic^7,Oic^8]-BK), apamin, charybdotoxin, glibenclamide, ruthenium red, methylene blue, atropine, tetraethylammonium and tetrodotoxin were infused over rabbit isolated corpus cavernosum tissues 20 min before and during a bolus injection of the appropriate agonists.

Effect of L-NAME and TRIM on rabbit cerebellum nitric oxide synthase activity in vitro

The *in vitro* actions of both L-NAME and TRIM were studied in a rabbit cerebellum homogenate by measuring their ability to inhibit the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described by Forstermann *et al.*, (1990). Briefly, the rabbits were anaesthetized with sodium pentobarbitone (Sagatal; 40 mg kg^{-1}, i.v.), the cerebella were rapidly removed and homogenized in five volumes of cold incubation buffer (50 mM Tris-HCl buffer, pH 7.4) containing 1 mM PMSF and 1 mM L-citrulline. The homogenates were incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$ and 10 mM L-arginine containing 100,000 d.p.m. of [2,3,4,5-^{3}H]-L-arginine monohydrochloride at room temperature (25–27°C). The NOS inhibitor, L-NAME or TRIM was then added to the homogenates to give a final concentration of 10 nM–3 mM. The protein content of the samples was determined according to the method of Peterson (1977) and the activities of cerebellar NOS are expressed as pmol L-citrulline produced min^{-1} mg^{-1} protein. The reduction in L-citrulline production caused by the inhibitors was expressed as a percentage of the maximum activity. From the semi-log concentration-activity curves, the values of pI_2($-\log_{10}$ of the molar concentration of inhibitor that causes 50% inhibition) were calculated.

Purification of Tityus serrulatus *venom*

Dried whole venom (400 mg) dissolved in ammonium bicarbonate buffer (0.01 M, pH 7.8) was purified with a 2.5 × 67.0 cm column of CM-cellulose-52 as previously described (Arantes *et al.*, 1989). The resulting pools, designated fractions I-XIII, were then directly lyophilized, wetted and again lyophilized until complete removal of the salt. All of these fractions were assayed in the rabbit isolated corpus cavernosum bioassay cascade. The doses of all fractions used to carry out the bioassay cascade were previously determined by calculating the extinction coefficient (ε) of each fraction. This coefficient gives the protein content (mg ml^{-1}) in a given absorbance. Usually, it is presented as the fraction absorbance which contains 1 mg ml^{-1} protein.

Drugs and Tityus serrulatus *venom*

The venom of *Tityus serrulatus* was provided by the Butantan Institute (São Paulo). The crude venom (lot no. 041088) was obtained by electrostimulation of the telsons of scorpions in captivity and was lyophilized and stored at −20°C.

Acetylcholine, apamin, aprotinin, atropine, D-arginine, L-arginine, bradykinin, calcitonin gene-related peptide, capsaicin, charybdotoxin, cromakalim, glibenclamide, indomethacin, methylene blue, N^{ω}-nitro-D-arginine methyl ester (D-NAME), N^{ω}-nitro-L-arginine methyl ester (L-NAME), noradrenaline, ruthenium red, substance P, tetraethylammonium, tetrodotoxin and vasoactive intestinal peptide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^{G}-iminoethyl-L-ornithine (L-NIO) was bought from Research Biochemicals International (Nattick, MA; U.S.A.). Glyceryl trinitrate (ampoules containing 1 mg ml^{-1} in isotonic saline) and pentobarbitone sodium (Sagatal) were acquired from Lipha Pharmaceuticals (London, U.K.) and May & Baker (Dagenham, Essex, U.K.), respectively. Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) was a gift from Hoechst AG (Frankfurt, Germany). 1-(2-Trifluoromethylphenyl) imidazole (TRIM) and 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ) were obtained from Tocris Cookson Inc. (St. Louis, MO, U.S.A.). [^{3}H]-L-arginine was purchased from Amersham International (U.K.).

Tityus serrulatus venom and test agents were stored in stock solution at −20°C and then diluted with isotonic saline (0.9% w/v) when assayed in the rabbit isolated corpus cavernosum strips.

The composition of the Krebs solution was (in mM): NaCl 118, $NaHCO_3$ 25, glucose 5.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.17 and $CaCl_2 6H_2O$ 2.5.

Statistical analysis

The relaxations induced by *Tityus serrulatus* venom and other agents were expressed relative to the submaximal relaxation induced by GTN, which was taken to be 100%. The results are shown as the mean ± s.e.mean of n experiments. Analysis of variance and Student's paired t test were employed to evaluate the data. A P value less than 0.05 was considered to indicate significance.

Results

Involvement of nitric oxide (NO) in the rabbit corpus cavernosum relaxations induced by Tityus serrulatus *venom*

Tityus serrulatus venom (3–100 μg), acetylcholine (ACh; 0.3–30 nmol), bradykinin (BK; 0.3–10 nmol) and glyceryl trinitrate (GTN; 0.5–10 nmol) caused dose-dependent relaxations of the rabbit isolated corpus cavernosum strips (not shown; $n=12$ each). The infusion of 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one] (ODQ; 30 μM, $n=4$), a selective inhibitor of NO-stimulated soluble guanylyl cyclase activity, abolished the relaxations induced by ACh (0.6 nmol; 66 ± 18% before and 1 ± 0.5% during ODQ infusion; $P<0.01$), BK (10 nmol; 49 ± 10% before and 0.0% during ODQ infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 85 ± 4% before and 5 ± 1% during ODQ infusion; $P<0.01$). The GTN (1.3 nmol)-induced relaxation was also significantly reduced by ODQ (98 ± 1% inhibition; $P<0.01$). The relaxations evoked by these agents were greatly restored 15 min after the ODQ infusion had stopped ($P<0.01$; Figure 1). An infusion of methylene blue (MB; 30 μM, $n=4$) significantly reduced the relaxations induced by ACh (0.6 nmol; 87 ± 3% before and 37 ± 11% during MB infusion; $P<0.05$) and *Tityus serrulatus* venom (10 μg; 96 ± 5% before and 33 ± 10% during MB infusion; $P<0.01$). The GTN (1.3 nmol)-induced relaxation was also significantly reduced by MB (32 ± 11% inhibition; $P<0.05$). However, in contrast to ODQ, the inhibitory effect of MB was irreversible (not shown).

The infusion of D-NAME (10 μM, $n=5$) did not affect either the basal tone of the rabbit isolated corpus cavernosum tissues or the relaxations induced by either ACh (0.6 nmol; 130 ± 18% before and 107 ± 14% during D-NAME infusion) or *Tityus serrulatus* venom (30 μg; 113 ± 16% before and 87 ± 18% during D-NAME infusion). However, the subsequent infusion of L-NAME (10 μM, $n=6$) further increased the tone of the rabbit isolated corpus cavernosum tissues and markedly reduced both ACh- (0.6 nmol; 107 ± 14% before and 17 ± 8% during L-NAME infusion; $P<0.01$) and *Tityus serrulatus* venom-induced relaxations (30 μg; 87 ± 18% before and 13 ± 7% during L-NAME infusion; $P<0.01$) without affecting those evoked by GTN. Infusion of L-arginine (300 μM, $n=6$), but not D-arginine (300 μM, $n=5$), partially reversed the increased tone and significantly restored the relaxations induced by ACh (0.6 nmol; 17 ± 8% before and 80 ± 13% during L-arginine infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 13 ± 7% before and 75 ± 12% during L-arginine infusion; $P<0.01$).

Similarly, the NO synthesis inhibitor N^G-iminoethyl-L-ornithine (L-NIO; 30 μM, $n=6$) increased the tone of the rabbit isolated corpus cavernosum and significantly reduced the relaxations induced by both ACh (0.6 nmol; 145 ± 27% before and 24 ± 6% during L-NIO infusion; $P<0.01$) and *Tityus serrulatus* venom (30 μg; 171 ± 26% before and 35 ± 10% during L-NIO infusion; $P<0.01$). At this dose, L-NIO had no significant effect on the GTN-induced relaxations. The subsequent infusion of L-arginine (300 μM, $n=6$) significantly restored both the increased tone and the rabbit isolated corpus cavernosum relaxations induced by ACh (110 ± 9%; $P<0.01$) and *Tityus serrulatus* venom (115 ± 17%; $P<0.01$) (Figure 2).

TRIM (1-(2-trifluoromethylphenyl) imidazole; 100 μM, $n=4$) had no effect on the rabbit isolated corpus cavernosum relaxations induced by *Tityus serrulatus* venom (30 μg; 90 ± 6% before and 85 ± 4% during TRIM infusion), ACh (0.6 nmol; 66 ± 18% before and 66 ± 18% during TRIM infusion), BK (10 nmol; 48 ± 11% before and 49 ± 11% during TRIM infusion) or GTN (1.3 nmol).

In vitro *NO synthase (NOS) activity*

The maximum NOS activity in the homogenates (in the absence of any inhibitor) was 5.96 ± 0.31 pmol L-citrulline min^{-1} mg^{-1} protein ($n=3$). When Ca^{2+} was omitted from the

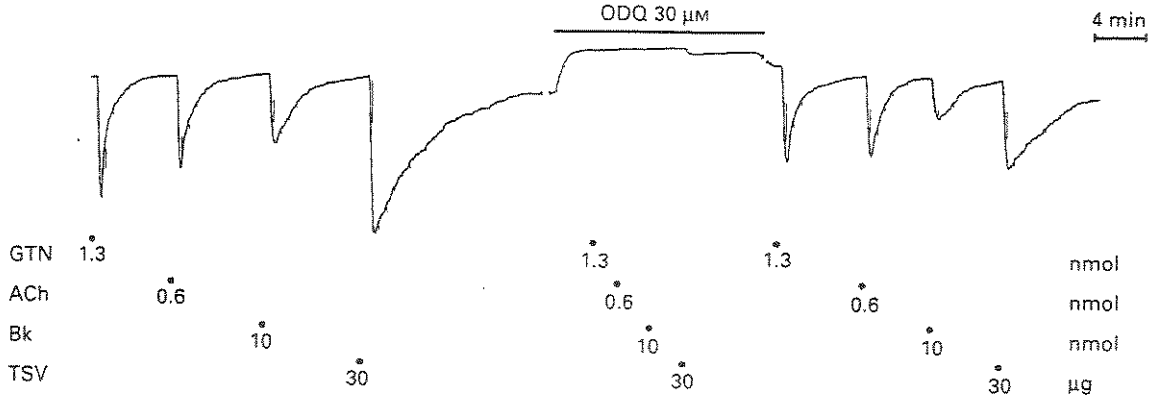

Figure 1 Inhibition by ODQ (30 μM) of the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol) and *Tityus serrulatus* venom (TSV; 30 μg). The infusion of ODQ increased the tone of the tissues and virtually abolished the relaxations induced by the above agonists. After the ODQ infusion had ended, the relaxations were greatly restored. This is a representative tracing of four experiments. Bk, bradykinin.

incubation media, the conversion of L-arginine to L-citrulline was inhibited by more than 90%, thus confirming that the enzymatic activity measured in the homogenates was due to calcium-dependent NOS. From the concentration vs NOS activity curves, the pI_2 values derived were 5.17 ± 0.76 and 2.62 ± 0.18 for L-NAME and TRIM, respectively ($n = 3$).

Lack of effect of aprotinin, Hoe 140 and atropine

The protease inhibitor aprotinin (Trasylol; 10 $\mu g\ ml^{-1}$) did not significantly affect the rabbit isolated corpus cavernosum relaxations induced by either ACh ($n = 4$) or *Tityus serrulatus* venom ($n = 4$; Table 1).

The stable bradykinin B_2 receptor antagonist Hoe 140 (50 nM), $n = 5$) virtually abolished the BK (10 nmol)-induced relaxations ($P < 0.01$) without affecting those induced by *Tityus serrulatus* venom (10 μg; Table 1).

The muscarinic receptor antagonist atropine (1 μM, $n = 5$) significantly reduced the ACh (0.6 nmol)-induced relaxation ($P < 0.01$) but had no effect on those induced by either BK (10 nmol) or *Tityus serrulatus* venom (10 μg; Table 1).

Effect of K^+ channel blockers

The ATP-dependent K^+ channel agonist cromakalim (10 and 30 nmol) caused dose-dependent (51 ± 9 and $95 \pm 19\%$ relaxation, respectively; $n = 6$) and long-lasting corpus cavernosum relaxations. Figure 3 shows that an infusion of the ATP-dependent K^+ channel antagonist glibenclamide (10 μM, $n = 6$) significantly reduced the cromakalim (30 nmol)-induced relaxations without affecting those induced by either ACh (0.6 nmol) or *Tityus serrulatus* venom (10 μg; Table 2 and Figure 3). In contrast, the cromakalim (30 nmol)-induced relaxations were not significantly affected by L-NAME (10 μM; $30 \pm 6\%$ before and $27 \pm 4\%$ during L-NAME infusion, $n = 4$).

The Ca^{2+}-activated K^+ channel blockers apamin (0.1 μM, $n = 6$) and charybdotoxin (0.1 μM, $n = 6$) also had no significant effect on the rabbit isolated corpus cavernosum relaxations induced by ACh, BK, cromakalim and *Tityus serrulatus* venom (Table 2). In addition, the K^+ channel blocker TEA (10 μM, $n = 4$) failed to affect ACh-, cromakalim- and *Tityus serrulatus* venom-induced relaxations (Table 2). At these concentrations, glibenclamide, apamin, charybdotoxin and TEA had no effect on the GTN-induced relaxations (not shown, $n = 6$).

Involvement of NANC mechanisms

Bolus injections of capsaicin (3 and 10 nmol) over the rabbit isolated corpus cavernosum tissues induced dose-dependent and non-tachyphylactic relaxations ($n = 6$) with a similar pattern to that induced by *Tityus serrulatus* venom and other agonists. The infusion of ruthenium red (RR; 30 μM, $n = 6$) did not significantly affect the relaxation induced by ACh (0.6 nmol; $88 \pm 20\%$ before and $83 \pm 13\%$ during RR infusion), BK (10 nmol; $49 \pm 9\%$ before and $55 \pm 8\%$ during RR infusion) and *Tityus serrulatus* venom (10 μg; $81 \pm 17\%$ before and $68 \pm 16\%$ during RR infusion). At this dose, RR markedly reduced the capsaicin-induced relaxations (Figure 4).

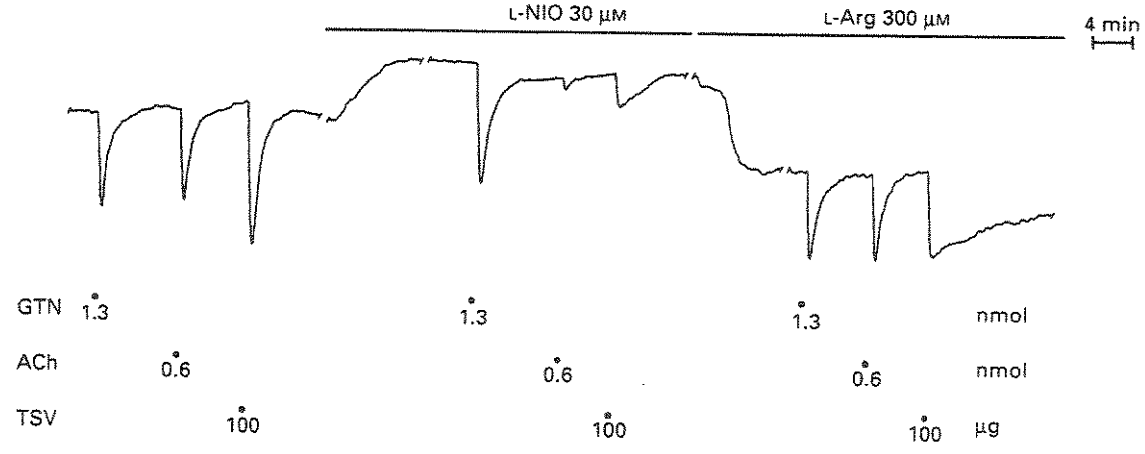

Figure 2 Effects of N^{ω}-nitro-imino-L-ornithine (L-NIO, 10 μM) and L-arginine (L-Arg; 300 μM) on rabbit corpus cavernosum (RbCC) strips. The infusion of L-NIO increased the RbCC tone and reduced the relaxation induced by both acetylcholine (ACh; 0.6 nmol) and *Tityus serrulatus* venom (TSV; 100 μg). The relaxations induced by glyceryl trinitrate (GTN; 1.3 nmol) were not significantly affected by the L-NIO infusion. Subsequent infusion of L-Arg reversed the increased RbCC tone and also partially restored the relaxations induced by ACh and TSV. This is a representative tracing of six experiments.

Table 1 Effect of aprotinin (10 $\mu g\ ml^{-1}$), Hoe 140 (50 nM) and atropine (1 μM) on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol), bradykinin (BK; 10 nmol) and *Tityus serrulatus* venom (TSV; 10 μg)

	RbCC relaxations (%)					
	ACh		*BK*		*TSV*	
Treatment	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
Aprotinin	141 ± 16	148 ± 46	ND	ND	84 ± 23	62 ± 34
Hoe 140	ND	ND	88 ± 5.5	16 ± 9*	98 ± 11	95 ± 10
Atropine	117 ± 7	10 ± 4*	56 ± 9	66 ± 18	95 ± 10	80 ± 6

Aprotinin, Hoe 140 and atropine were infused over the RbCC tissues at a flow rate of 0.1 ml min^{-1} for at least 20 min before injection of the agonists. The RbCC relaxations induced by ACh, BK and TSV were expressed (mean $\pm$ s.e.mean, $n = 4-5$) relative to the submaximal relaxation induced by glyceryl trinitrate which was taken to be 100%. ND, not determined. * $P < 0.01$, compared to the respective control.

The bolus injection of substance P (SP; 0.75 and 2.2 nmol) had no effect on the rabbit isolated corpus cavernosum tissues ($n=4$, not shown), although higher doses (7.5 nmol) evoked a short-lived contraction (10.5±2%, $n=4$) of the tissues. In doses up to 2 nmol, calcitonin gene-related peptide (CGRP) had no significant effect on the rabbit isolated corpus

Figure 3 The ATP-dependent K^+ channel antagonist glibenclamide (10 μM) virtually abolished the rabbit corpus cavernosum (RbCC) relaxation induced by the K^+ channel opener cromakalim (30 nmol) without affecting that induced by *Tityus serrulatus* venom (10 μg). The RbCC relaxations induced by acetylcholine (ACh; 0.6 nmol) and glyceryl trinitrate (GTN; 1.3 nmol) were also not affected by glibenclamide. This is a representative tracing of six experiments.

Table 2 Lack of effect of K^+ channel blockers on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh; 0.6 nmol), bradykinin (BK; 1 nmol), cromakalim (CMK; 30 mnol) and *Tityus serrulatus* venom (TSV; 10 μg)

	RbCC relaxations (%)							
	Glibenclamide		*Apamin*		*Charybdotoxin*		*TEA*	
Agents	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ACh	112±11	98±8	99±7	90±8	107±18	100±20	64±13	80±11
BK	ND	ND	47±3	48±13	44±9	43±10	ND	ND
CMK	95±19	11±6*	57±15	67±21	ND	ND	48±8	46±13
TSV	125±11	102±11	83±13	88±11	84±20	87±19	60±9	69±14

Glibenclamide (10 μM), apamin (0.1 μM), charybdotoxin (0.1 μM) and tetraethylammonium (TEA, 10 μM) were infused over the RbCC tissues at a flow rate of 0.1 ml min^{-1} for at least 20 min before injection of the agonists. The RbCC relaxations induced by ACh, BK and TSV were expressed (mean±s.e.mean, $n=6$) relative to the submaximal relaxation induced by glyceryl trinitrate which was taken to be 100%. ND, not determined. * $P<0.05$ compared to the respective control.

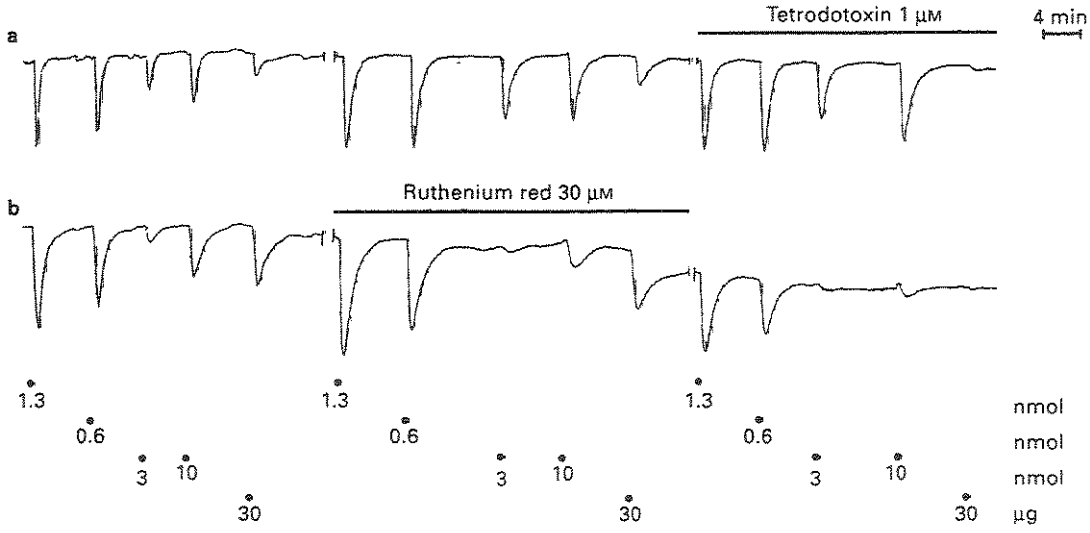

Figure 4 Effects of ruthenium red and tetrodotoxin on rabbit corpus cavernosum (RbCC). The infusion of ruthenium red (30 μM) over the second tissue (b) markedly inhibited the capsaicin-induced RbCC relaxations, without affecting those evoked by *Tityus serrulatus* venom. The sodium channel blocker tetrodotoxin (1 μM) infused over both tissues abolished the RbCC relaxations induced by *Tityus serrulatus* venom (TSV; 30 μg), but had no effect on those induced by capsaicin (Cap; 3 and 10 nmol), acetylcholine (ACh; 0.6 nmol) and glyceryl trinitrate (GTN; 1.3 nmol). This is a representative tracing of six experiments.

cavernosum tissues ($n=4$, not shown). Bolus injection of vasoactive intestinal peptide (VIP; 0.6 and 2 nmol) caused small relaxations ($8\pm2.5\%$ and $19\pm4\%$, respectively). The VIP (2 nmol)-induced relaxations were not significantly affected by L-NAME (10 μM; $16\pm6\%$ before and $14\pm5\%$ during L-NAME infusion; $n=4$, not shown).

Effect of the sodium channel blocker tetrodotoxin

An infusion of tetrodotoxin (TTX; 1 μM, $n=6$) virtually abolished the *Tityus serrulatus* venom (30 μg)-induced relaxations ($66\pm9\%$ before and $4\pm2\%$ during TTX infusion; $P<0.01$). At this concentration, TTX did not affect either the tone of the rabbit isolated corpus cavernosum tissues or the relaxations induced by capsaicin (10 nmol; $71\pm6\%$ before and $68\pm6\%$ during TTX infusion, $n=6$), BK (10 nmol; $75\pm9\%$ before and $73\pm7\%$ during TTX infusion), ACh (0.6 nmol; $79\pm1\%$ before and $90\pm10\%$ during TTX infusion) and GTN (1.3 nmol) (Figure 4). In addition, the established relaxations by *Tityus serrulatus* venom were promptly reversed by the same concentration of TTX (Figure 5).

Bioassay cascade of fractions purified from Tityus serrulatus *venom*

The bioassay cascade of the fractions I-XIII revealed that fractions I-IX and XIII (up to 80 μg) had no effect on the rabbit isolated corpus cavernosum tissues (not shown; $n=6$). In contrast, fractions X (1.2–12 μg), XI (1.4–14 μg) and XII (1–10 μg) caused dose-dependent relaxations. These fractions corresponded to 4.01, 3.58 and 2.97% of whole venom, respectively. *Tityus serrulatus* venom (30 μg; $n=8$) caused $65\pm8\%$ whereas fraction X (3.8 μg, $n=8$), fraction XI (4.2 μg, $n=8$) and fraction XII (3.0 μg, $n=8$) caused $44\pm8\%$, $56\pm12\%$ and $54\pm10\%$ of relaxation, respectively. The infusion of either L-NAME (10 μM; $n=4$) or TTX (1 μM; $n=4$) reduced by >90% the relaxations of rabbit isolated corpus cavernosum induced by these fractions.

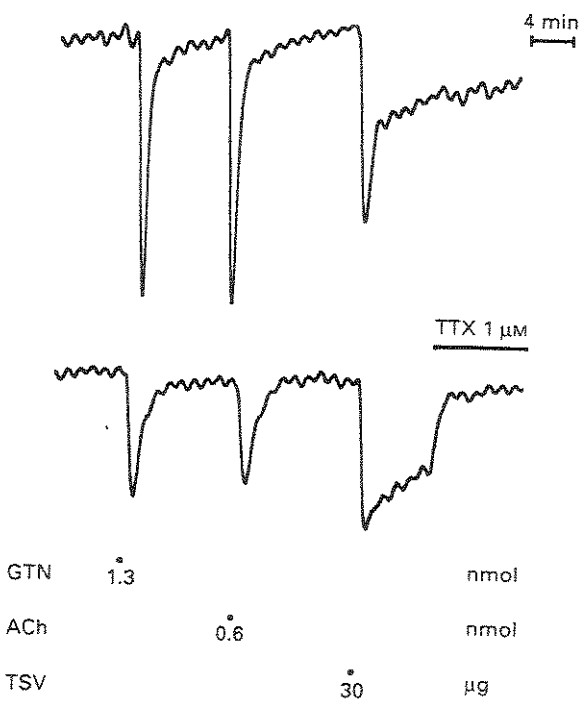

Figure 5 Infusion of the sodium channel blocker tetrodotoxin (TTX, 1 μM) over the second tissue promptly reversed the established RbCC relaxation induced by *Tityus serrulatus* venom (TSV; 30 μg). This is a representative tracing of four experiments.

Discussion

Our results indicate that *Tityus serrulatus* scorpion venom relaxes the rabbit corpus cavernosum via the release of NO. The involvement of NO was confirmed by the findings that the non-specific NOS inhibitors L-NAME (Moore *et al.*, 1989) and L-NIO (Rees *et al.*, 1990) markedly reduced the *Tityus serrulatus* venom-induced relaxations and that this inhibition was reversed by L-arginine (but not D-arginine). Methylene blue (Gruetter *et al.*, 1981; Rapoport & Murad, 1983) and ODQ (Garthwaite *et al.*, 1995), inhibitors of soluble guanylate cyclase, also markedly reduced the relaxation induced by *Tityus serrulatus* venom, further supporting the involvement of NO. In contrast to MB, the inhibition by ODQ was reversible. Whether this finding reflects a weaker binding of the inhibitor to soluble guanylate cyclase remains to be established.

NO activates Ca^{2+}-dependent K^+ channels in rabbit aorta (Bolotina *et al.*, 1994). Cromakalim relaxed the rabbit isolated corpus cavernosum and this effect was selectively blocked by glibenclamide, indicating the presence of functional ATP-dependent K^+ channels in the erectile tissue. However, the finding that *Tityus serrulatus* venom-induced relaxations were not affected by either the Ca^{2+}-dependent K^+ channel antagonists, apamin (Burgess *et al.*, 1980) and charybdotoxin (Gimenez-Gallego *et al.*, 1986), or by glibenclamide, excludes the involvement of these K^+ channels in the venom transduction mechanism. The failure of TEA to affect venom-induced relaxations further supports the contention that K^+ channels do not play a role.

Similar to the scorpion venom, *Phoneutria nigriventer* spider venom also relaxes rabbit isolated corpus cavernosum via the release of NO. However, this release is secondary to tissue kallikrein activation (Lopes-Martins *et al.*, 1994). Since neither the protease inhibitor aprotinin (Vogel & Werle, 1970) nor the BK antagonist Hoe 140 (Wirth *et al.*, 1991) affected the *Tityus serrulatus* venom-induced relaxation, we may rule out this intermediate step in NO release.

The erectile tissues from different animal species are innervated by both adrenergic excitatory and cholinergic inhibitory nerve fibres (see Andersson & Wagner, 1995). The failure of the muscarinic receptor antagonist atropine to affect the *Tityus serrulatus*-induced relaxations indicates that the venom does not act either by activating cholinergic fibres nor through the presence of ACh-like substances in the venom itself.

The erectile tissues are also richly innervated by NANC inhibitory nerve fibres (Gillespie, 1972; Klinge & Sjostrand, 1974) and this is believed to play a pivotal role in the neural mechanisms involved in penile erection through the release of NO (Ignarro *et al.*, 1990; Pickard *et al.*, 1991; Kim *et al.*, 1991; Rajfer *et al.*, 1992). Thus, the potential sources of NO production in the rabbit isolated corpus cavernosum preparation employed in this study are both NANC neurones and the endothelium, which covers the network of sinusoidal capillaries supplying the cavernosal tissue.

The classical sodium channel blocker tetrodotoxin specifically inhibited the *Tityus serrulatus* venom-induced rabbit isolated corpus cavernosum relaxations, strongly indicating that NO release by the venom is preceeded by nerve activation, possibly involving the NANC system. Indeed, the addition of *Tityus serrulatus* venom (or tityustoxin) to the peripheral cut

end of either sciatic or saphenous nerve of the rat releases a neurogenic permeability factor from sensory fibres, which is responsible for the increase in vascular permeability and oedema formation in the areas supplied by the nerves (Garcia-Leme *et al.*, 1977).

NANC activation leads to the release of neuropeptides such as substance P, CGRP and VIP. Capsaicin, a substance known to activate primary sensory neurones (Jancsó *et al.*, 1977) causes the release of these neuropeptides from NANC terminals in different tissues (Holzer, 1991). However, it is unlikely that the venom activates NANC primary sensory C fibres to cause the release of neuropeptides, since bolus injections of substance P and CGRP were unable to relax rabbit isolated corpus cavernosum tissues whereas VIP caused small relaxations. The observation that capsaicin caused NO-independent and tetrodotoxin-insensitive relaxations reinforces the concept, that rabbit isolated corpus cavernosum relaxation by *Tityus serrulatus* venom does not result from the activation of capsaicin-sensitive sensory neurones. Furthermore, ruthenium red, an inhibitor of capsaicin-induced stimulation of sensory neurones (Buckely *et al.*, 1990; Amann & Maggi, 1991), markedly reduced capsaicin-induced relaxations but failed to affect those induced by the venom. The mechanism by which capsaicin relaxes the rabbit isolated corpus cavernosum is not yet clear but it may involve a direct vasorelaxing effect on the cavernosal tissues, independent of both endothelium and nerve stimulation.

We propose therefore that *Tityus serrulatus* venom acts selectively on NANC fibres, possibly nitrergic neurones, and that the NO generated in the nerve diffuses through the nerve endings to relax the adjacent vascular smooth muscle. Indeed, neuronal NOS (bNOS) has been detected in both the rat (Burnett *et al.*, 1992) and human (Burnett *et al.*, 1993) penis by use of a specific NOS antibody and immunohistochemistry. The findings that TRIM, a specific neuronal NOS inhibitor in the mouse (Handy *et al.*, 1995), did not affect the NO release induced by *Tityus serrulatus* venom, may reflect its reduced potency on the rabbit enzyme.

Several protein toxins purified from South American scorpions venoms have been described and classified either as α or β toxins, according to their effects on voltage-dependent Na^+ channels of excitable cells (Barhanin *et al.*, 1982). The former prolong the action potential, whereas the latter do not depend on the membrane potential for to their action. Interestingly, a peptide isolated from fraction XI (MW 7230) delays the inactivation of Na^+ channels of the B fibres of the rabbit vagus nerve (Arantes *et al.*, 1993), whereas fraction XIII, which does not cause rabbit isolated corpus cavernosum relaxation, contains a β toxin (Jonas *et al.*, 1986). Thus, our results indicate that activation of Na^+ channels in NANC fibres is essential for NO release.

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Development of cardiomyocyte hypotrophy in rats under prolonged treatment with a low dose of a nitric oxide synthesis inhibitor

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Abstract

Chronic administration of the nitric oxide (NO) synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to rats causes hypertension and morphological abnormalities in the heart, consisting mainly of ventricular hypertrophy and foci of necrosis and fibrosis. Since these phenomena have usually been described with high (or moderate) doses of L-NAME, this study was undertaken to evaluate the effects of a low dose of L-NAME on arterial blood pressure, heart weight index, left ventricular weight index, amount of ventricular fibrosis, and cardiomyocyte size. Male Wistar rats received L-NAME (7.5 mg/kg per day) in the drinking water for 2, 4, and 6 months, whereas control animals received tap water alone. At this dose, L-NAME caused 90% inhibition ($P < 0.001$) of brain NO synthase (NOS) activity. The chronic L-NAME treatment caused an approximately 15% reduction in body weight of the animals, and no death was observed. The tail–cuff pressure was markedly ($P < 0.01$) elevated in L-NAME-treated rats. A significant ($P < 0.05$) reduction in both heart weight index (13–20% decrease) and left ventricular weight index (20–34% decrease) at 2, 4, and 6 months of treatment was observed in L-NAME-treated rats. The cardiomyocyte size in subendocardial, subepicardial, and midmyocardial regions of the left ventricles was time-dependently reduced, irrespective of the region studied, as measured at 2 (11% decrease), 4 (28% decrease, $P < 0.05$), and 6 (45% decrease, $P < 0.05$) months of chronic L-NAME treatment. The amount of fibrous tissue was unaltered at 2 and 4 months, but a small (but significant) increase in the amount of fibrous tissue was detected at 6 months (7.1 ± 0.2 %, $P < 0.05$) compared to that of control animals (5.9 ± 0.2%). Our results show that chronic treatment of rats with a low dose of L-NAME for prolonged periods (up to 6 months) causes arterial hypertension accompanied by significant reductions in heart weight, left ventricular weight indexes, and cardiomyocyte size.

Keywords: N^{ω}-nitro-L-arginine methyl ester; Heart lesion; Ventricular hypertrophy; Stereology

1. Introduction

Nitric oxide (NO) plays a major role in modulating regional blood flow and arterial blood pressure in different animal species, including humans (Moncada et al., 1991). Former studies reported that daily administration of NO synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) caused marked and sustained arterial hypertension in rats (Baylis et al., 1992; Ribeiro et al., 1992). A number of subsequent studies reported the use of prolonged ingestion of NOS inhibitors to evaluate physiopathological changes mediated by NO in different rat models (see Zatz and Baylis, 1998).

The cardiovascular studies, in which rats ingested L-NAME daily, mainly used relatively high (70–250 mg kg^{-1} day^{-1}) or moderate (40–50 mg kg^{-1} day^{-1}) doses of NOS inhibitors for a period of time varying from 1 to 8 weeks. Using this range of doses, hypertension induced by chronic L-NAME intake may be accompanied by marked pathological changes in both heart (Jover et al., 1993; Rhaleb et al., 1994; Moreno-Jr et al., 1995, 1996) and kidney (Fujihara et al., 1994), as well as in arterial vessels (Delacretaz et al., 1995; Numaguchi et al., 1995; Babal et al., 1997; Chillon et al., 1997; Moreau et al., 1998). In the heart, morphological abnormalities consist mainly of ventricular hypertrophy and foci of necrosis and fibrosis (Numagachi et al., 1995; Moreno-Jr et al., 1996; Babal et al., 1997; Akuzawa et al., 1998; Devlin et al., 1998;

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K-Laflamme et al., 1998; Luvara et al., 1998). However, the mechanism by which these alterations take place in the heart is unclear. Although concomitant ingestion of antihypertensive agents prevents L-NAME-induced hypertension, the resulting hypertrophy and cardiac lesions can either be unaltered (Moreno-Jr et al., 1995) or attenuated by these agents (Numagachi et al., 1995; Oliveira et al., 1999). The purpose of this study was to further understand the relationship between high levels of blood pressure, changes of cardiomyocyte size, and ventricular lesions in response to chronic L-NAME treatment. We therefore treated rats chronically with a low dose of L-NAME (7.5 mg kg^{-1} day^{-1}), and evaluated hypertension, heart weight index, left ventricular weight index, ventricular fibrosis, and cardiomyocyte size after 2, 4, and 6 months of treatment.

2. Material and methods

2.1. Animals

Male Wistar rats (approximately 150 g at the beginning of the study) were provided by the Central Animal House-State University of Campinas (CEMIB-UNICAMP). The animals were maintained under light and temperature-controlled conditions (12 h day/12 h night, 25°C) and were fed with a standard chow (Nuvilab CR-1®, Nuvital Nutrientes, Curitiba, Brazil). All experiments were in accordance with the guidelines of the UNICAMP for animal care.

2.2. Treatment of the animals with L-NAME

The chronic treatment with L-NAME was performed as previously described (Ribeiro et al., 1992). The animals received L-NAME dissolved in the drinking water to give a daily dose of 7.5 mg kg^{-1} day^{-1}. The animals were killed at 2 ($n = 10$), 4 ($n = 10$), and 6 ($n = 15$) months after treatment. Control animals receiving tap water alone were used alongside each experimental group ($n = 10–15$). The concentration of L-NAME in the water would give a total amount of 2 mg rat^{-1} day^{-1}. This was maintained constant; so in the beginning, the rat would have a higher intake than 7.5 mg/kg (first 2 months) and at the end of the experiment (last month), the total dose would be slightly lower than 7.5 mg/kg. The concentration in the water was calculated by our previous experience in the daily intake of water per rat.

2.3. Cardiac weight indices

At the end of the study, the animals were killed with an overdose of sodium pentobarbital (Sagatal®), and the heart was dissected out and washed with saline (0.9%, w/v). The hearts were fixed in a 10% formalin for 24 h. Heart weight was obtained by the removal of both atria, and left ventricular weight was determined by excising the right ventricle and weighing the remaining tissue. Finally, heart weight and left ventricular weight indices were calculated by dividing heart weight and left ventricular weight by body weight measured in the last week of treatment.

2.4. Stereological procedures

Stereological analysis was performed according to the method described by Aherne (1970). For this procedure, formalin-fixed left ventricle and septum were cut into five equidistant rings perpendicular to the long axis of the ventricle. The rings were then embedded in paraffin, and 5-μm sections were stained with Masson's trichrome. Analysis of the slides was performed blindly using a light microscope (Zeiss, Germany), and the relative volume occupied by each element of the ventricle (myocardial fibers and fibrous tissue) was measured with a special ocular containing a 25-point reticulum (five parallel lines with five points each, kpl 8×, Zeiss). For counting, 50 microscopic fields were evaluated and the relative volume (Ppi) occupied by each component was calculated as follows: $Ppi = p/(P - R)$, where p is the number of reticular points hitting each cardiac element, P is the total number of reticular points and R is the number of points hitting artefactual retraction areas. To determine the cardiomyocyte size, the cell diameters were measured by using a light optical system supplied with a graduated eyepiece micrometer and a 40× objective (400 magnification). Fifteen cells, randomly selected from the subepicardial, midmyocardial, and subendocardial regions, were measured for each animal from the different experimental groups.

2.5. Determination of NOS activity in brain

Determination of NOS activity in brains from the both control and L-NAME-treated rats was carried out according to a method previously described, which is based on the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (Förstermann et al., 1990). For this purpose, the brains from the control and L-NAME-treated rats were rapidly removed, weighed, and individually homogeneized in five volumes of cold incubation buffer (Tris–HCl 50 mM, pH 7.4) containing 1 mM of phenyl methyl sulphonyl fluoride and 1 mM of L-citrulline. The homogenates were incubated at room temperature for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$, and 10 μM L-arginine containing 100,000 dpm of L-[2,3,4,5-^{3}H]arginine monohydrochloride. The determination of NOS activity was also performed in the absence of calcium (omission of $CaCl_2$ and addition of 1 mM EGTA). Protein content of the samples was determined (Peterson, 1977), and brain NOS activity is expressed as picomoles of L-citrulline produced per minute and per milligram of protein.

Table 1
Body weight and tail–cuff pressure in animals treated chronically with L-NAME (7.5 mg/kg per day) and in control animals that received tap water alone
The results represent the means ± S.E.M. for 10–15 rats.
BW, body weight; TCP, tail–cuff pressure.

Months	BW (g)		TCP (mm Hg)	
	Control	L-NAME	Control	L-NAME
2	306 ± 8.4	259 ± 10.5*	122 ± 0.6	151 ± 1.0*
4	341 ± 7.8	291 ± 11.0*	118 ± 1.7	151 ± 1.2*
6	396 ± 7.3	341 ± 11.3*	120 ± 0.7	146 ± 2.0*

* $P < 0.05$ compared to control values.

2.6. Blood pressure measurements

The arterial blood pressure was evaluated weekly. For each animal, mean blood pressure was measured at least in triplicate by a tail–cuff method (Zatz, 1990). Briefly, a small electret microphone, used as a sensor, was connected to the tail by a piece of rubber tubing. This design provides selective attenuation of tail pulsations appearing as the cuff is deflated between systolic and mean arterial pressures. Mean, rather than systolic pressure, appears to be evaluated in the conscious rat with this method.

2.7. Drugs

L-NAME and pentobarbital sodium (Sagatal®) were purchased from Sigma (USA) and May & Baker (UK), respectively. L-[2,3,4,5-^{3}H] arginine (specific activity 60.0 Ci/mmol) was supplied by Amersham (UK). The reagents to measure brain NOS activity were purchased from Sigma.

2.8. Data and statistical analysis

Results are expressed as mean ± S.E.M. Analysis of variance (ANOVA) followed by Bonferroni test was applied in order to assess the differences in body weight and tail–cuff pressure. For stereological procedures, ANOVA was followed by Tukey test. A P-value < 0.05 was considered significant.

3. Results

3.1. Body weight and survival

L-NAME (7.5 mg kg^{-1} day^{-1}) did not significantly affect the body weight of the animals until the fourth week of treatment (249 ± 1.9 and 237 ± 8.1 g, for control and treated, respectively; $n = 10$), after which, a small (but significant) reduction in body weight of approximately 15% was observed in the L-NAME-treated animals ($P < 0.05$; Table 1). All the animals from the control (tap water) and L-NAME groups survived.

3.2. Tail–cuff pressure

L-NAME caused a marked increase ($P < 0.01$) in tail–cuff pressure, reaching submaximal values after 1 month of treatment (142 ± 2.1 mm Hg; $P < 0.01$) compared to that of control animals (120 ± 2.7 mm Hg). In this group of animals, tail–cuff pressure remained significantly ($P < 0.01$) elevated for the whole period of treatment with L-NAME (Table 1). Animals receiving tap water alone had no significant changes in tail–cuff pressure (Table 1).

3.3. Cardiac weight indices

Significant reductions in heart weight index and left ventricular weight index at 2, 4, and 6 months, were observed in the L-NAME-treated animals compared to the control group (Table 2).

3.4. Stereological analysis in subendocardial, subepicardial, or midmyocardial regions: cardiomyocyte size and fibrous tissue

Animals receiving L-NAME showed a marked and progressive decrease in cardiomyocyte size at 4 and 6 months after treatment (Fig. 1). This reduction of cardiomyocyte size was of the same magnitude, irrespective of the region of the left ventricle studied (subendocardial, subepicardial, or midmyocardial) and was clearly detected at 4 (25–30%

Table 2
Heart weight index (HWI), left ventricular weight index (LVWI) and amount (%) of fibrous tissue in animals treated chronically with L-NAME (7.5 mg/kg per day) for 6 months and in control animals that received tap water alone
The results represent the means ± S.E.M. for 10–15 rats for each group.

Months	HWI		LVWI		Fibrous tissue (%)	
	Control	L-NAME	Control	L-NAME	Control	L-NAME
2	2.30 ± 0.07	1.82 ± 0.06*	2.25 ± 0.12	1.80 ± 0.12*	5.7 ± 0.1	5.7 ± 0.2
4	2.90 ± 0.03	1.90 ± 0.10*	1.83 ± 0.03	1.59 ± 0.08*	5.9 ± 0.3	5.7 ± 0.2
6	2.98 ± 0.04	1.95 ± 0.09*	1.87 ± 0.02	1.50 ± 0.08*	5.9 ± 0.2	7.1 ± 0.2*

* $P < 0.05$ compared to control values.

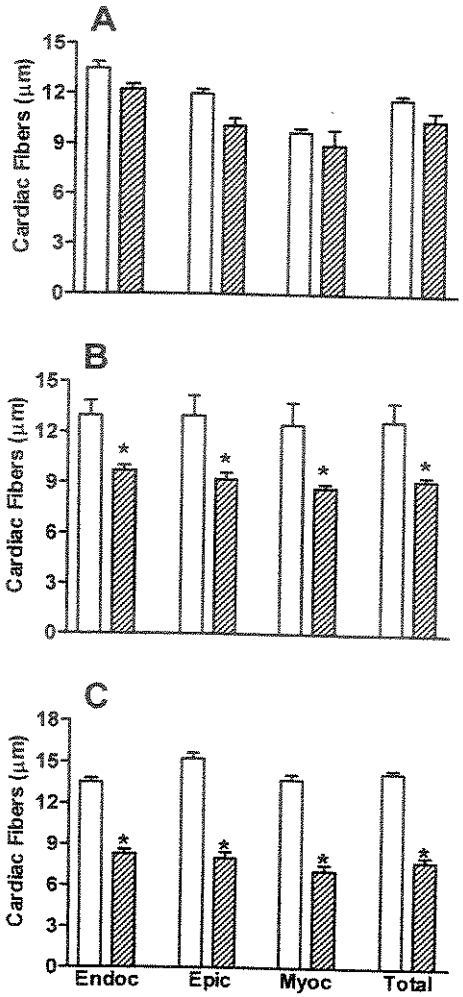

Fig. 1. Cardiomyocyte size (μm) in subendocardial, subepicardial, and midmyocardial regions of the left ventricle from rats treated chronically with L-NAME (7.5 mg/kg per day) for 2 (Panel A), 4 (Panel B), and 6 (Panel C) months. Control animals that received tap water alone are shown by the open columns, whereas L-NAME-treated animals are shown by the hatched columns. $^{*}P < 0.05$ compared to control values. Endoc, subendocardial; Epic, subepicardial; Myoc, midmyocardial.

reduction) and 6 (40–50% reduction) months of chronic L-NAME treatment (Fig. 1).

For measurement of fibrous tissue in the left ventricle, we assumed the amount of fibrous tissue to equal the sum of postnecrotic fibrous scars and interstitial and perivascular fibrosis. With L-NAME treatment, the amount of fibrous tissue was unaltered at 2 and 4 months, but a small (but significant; $P < 0.05$) increase in fibrous tissue was observed at 6 months of treatment (Table 2).

3.5. Brain NOS activity

The brain NOS activity in rats treated chronically with L-NAME for 2 months was markedly reduced (0.59 ± 0.10 pmol citrulline min^{-1} mg^{-1}; $n = 3$) compared to that of control animals (5.04 ± 0.48 pmol min^{-1} mg^{-1}; $n = 3$; $P < 0.01$). The omission of Ca^{2+} and addition of EGTA to the brain homogenates abolished the NOS activity in both the control (0.6 ± 0.1 pmol citrulline min^{-1} mg^{-1}; $n = 3$) and L-NAME-treated animals (0.1 ± 0.06 pmol citrulline min^{-1} mg^{-1}; $n = 3$); thus, indicating that conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was due to constitutive NOS.

4. Discussion

Our results show that chronic treatment of rats with a low dose of L-NAME (7.5 mg kg^{-1} day^{-1}) for prolonged periods (4 and 6 months) causes significant hypertension accompanied by significant reductions in both heart weight and left ventricular weight indexes as well as in cardiomyocyte size. This finding contrasts with the literature, since in other studies (usually using higher doses of L-NAME for shorter periods), ventricular hypertrophy and increase in cardiomyocyte size were reported in association with hypertension (Numagachi et al., 1995; Moreno-Jr et al., 1996; Devlin et al., 1998; Gomes-Pessanha et al., 1999).

The reduction in cardiomyocyte size is not due to the sustained hypertension induced by L-NAME, since it usually leads to ventricular hypertrophy in animals (Dussaule et al., 1986) and humans (Hammond et al., 1988; Levy et al., 1989) in response to the increased afterload (Dominiczak et al., 1997). This discrepancy indicates that cardiac hypotrophy in NO-deficient rats is not due to mechanical overload. Two possible explanations for these results can be proposed: one, based on a systemic deficiency of NO, leading to a decrease in blood supply to the heart muscle; and the other, based on a local deficiency of NO, causing metabolic changes in the cardiomyocyte itself.

The first hypothesis is supported by the findings that ventricular lesions caused by higher doses of L-NAME appear to be mainly the consequence of extensive myocardial ischaemia, which ultimately leads to cardiomyocyte death, necrosis, and subsequent formation of interstitial fibrosis (Moreno-Jr et al., 1996). These phenomena take place independently of arterial hypertension since prolonged treatment of rats with angiotensin-converting enzyme inhibitors (Hropot et al., 1994; Pechanova et al., 1997; Akuzawa et al., 1998; Matsubara et al., 1998) reduces hypertension but fails to affect the accompanying ventricular lesions (Moreno-Jr et al., 1995). Therefore, the reduction of cardiomyocyte size, as evidenced in this study, could represent a prior step before cardiomyocyte death. Thus, a slow (but persistent) reduction in coronary flow in response to a low dose of L-NAME would lead to an inadequate supply of oxygen and impaired myocardial contractility, resulting in cardiomyocyte thinning and, in later stages, in myocyte death and replacement by fibrous tissue. Consistent with this, a significant increase in fibrous tissue was observed 6 months after L-NAME treat-

ment. It is likely that hypotrophy of cardiomyocytes with higher doses of L-NAME was undetectable as intense coronary ischaemia would occur promptly after L-NAME administration, thereby accelerating cardiomyocyte death. Indeed, cardiac infarction, induced by high doses of L-NAME, is observed as soon as 72 h after oral administration (Moreno-Jr et al., 1997).

Mechanical (stretch) and humoral factors (thyroid hormones, catecholamines, and the renin–angiotensin system hormones) are known to regulate the growth of adult hearts (see Hudlicka and Brown, 1996). However, little is known about whether NO is involved in cardiomyocyte growth (Pignatti et al., 1999). Previous studies demonstrated that cardiomyocytes produce NO (Kitakaze et al., 1995) and express both type II (Pinsky et al., 1995; Buchwalow et al., 1997) and type III (Stein et al., 1998) NOS. Since the low dose of L-NAME caused hypotrophy of cardiomyocytes (and inhibited NOS), it is possible that NO has hypertrophic actions in this particular cell type. Indeed, NO has been shown to have proliferative actions in both endothelial cells from coronary postcapillary venules (Ziche et al., 1994) and chick cardiomyocytes in culture (Pignatti et al., 1999).

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The role of nitric oxide on the relaxations of rabbit corpus cavernosum induced by *Androctonus australis* and *Buthotus judaicus* scorpion venoms

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Abstract

In this study, we have investigated the relaxing effects of both *Androctonus australis* venom (AAV) and *Buthotus judaicus* venom (BJV) on the rabbit corpus cavernosum (RbCC) smooth muscle strips. The RbCC strips were mounted in a cascade system and superfused with warmed and gassed Krebs solution. The nitric oxide (NO) synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10 μM), but not D-NAME (10 μM), significantly inhibited the RbCC relaxations induced by acetylcholine (ACh, 0.6 nmol), AAV (30 μg) and BJV (30 μg). Subsequent infusion of L-arginine (300 μM), but not of D-arginine (300 μM), partially restored the relaxations evoked by these agents. The brain NO synthase inhibitor 7-nitroindazole (7-NI, 10 μM) also inhibited the relaxant responses elicited by the scorpion venoms. The guanylyl cyclase inhibitors methylene blue (MB, 30 μM) and 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ, 10 μM) virtually abolished the relaxations induced by either AAV or BJV. The infusion of muscarinic receptor antagonists such as scopolamine and atropine (1 μM, each) completely abolished the ACh-induced relaxations but had no effect on those evoked by the scorpion venoms. The Na^+ channel blocker tetrodotoxin (1 μM) prevented the relaxations evoked by both AAV and BJV. Thus, NO released from nitrergic nerve fibres mediates the relaxations elicited by AAV and BJV in the rabbit cavernosal tissue.

Keywords: *Androctonus australis*; *Buthotus judaicus*; Nitric oxide; Priapism; Tetrodotoxin

1. Introduction

Scorpion venoms exert a variety of effects on excitable tissues, due to their action at the peripheral nervous system enhancing the release of neurotransmitters (see Freire-Maia and Campos, 1989). The clinical symptomatology observed in severe scorpion envenomation involves mainly sympathetic (tachycardia, hypertension, sweating and mydriasis) and parasympathetic (bradycardia, hypotension, secretions and miosis) stimulation as well as central manifestations such as irritability, hyperthermia, vomiting, tremor and convulsion. The occurrence of priapism is also a common sign of scorpion envenomation, especially in accidents involving scorpions of Buthinae family, particularly the genus *Buthus* and *Leiurus* (Bawaskar, 1982; Amitai et al., 1985; Hershkovich et al., 1985). The mechanisms involved in venom-induced priapism are unclear and little studied. In the rat isolated anococcygeus muscle, a persistent activation of peripheral NANC nerves by *Leiurus quinquestriatus quinquestriatus* scorpion venom leads to nitric oxide (NO) release (Gwee et al., 1995). Recently, we demonstrated that *Tityus serrulatus* scorpion venom (TSV) relaxes the rabbit corpus cavernosum (RbCC) due to the release of NO from nitrergic non-adrenergic non-cholinergic (NANC) nerve terminals (Teixeira et al., 1998). NO released from NANC inhibitory nerve fibres has been shown to play a pivotal role in the neural mechanisms involved in penile erection (Ignarro et al., 1990; Kim et al., 1991; Pickard et al., 1991;

Abbreviations: AAV, *Androctonus australis* venom; BJV, *Buthotus judaicus* venom; RbCC, Rabbit corpus cavernosum; NANC, Non-adrenergic non-cholinergic; NO, Nitric oxide; NOS, Nitric oxide synthase

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Rajfer et al., 1992). Since studies on the effects of scorpion venoms on NANC neurotransmission and cavernosal relaxation are generally lacking, the present study was designed to examine the relaxant effects of venoms *Androctonus australis* and *Buthotus judaicus*, two scorpions belonging to Buthinae family, on the isolated cavernosal smooth muscle. We have focussed our attention mainly on the role of NO mediating the scorpion venom-induced cavernosal relaxations.

2. Material and methods

2.1. Venoms and reagents

Acetylcholine, D-arginine, L-arginine, atropine, indomethacin, methylene blue, N^{ω}-nitro-D-arginine methyl ester, N^{ω}-nitro-L-arginine methyl ester, 7-nitroindazole (7-NI), noradrenaline, 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ), scopolamine, tetrodotoxin, *Androctonus australis* venom (AAV) and *Buthotus judaicus* venom (BJV) were acquired from Sigma Chemical Co (St. Louis, USA). Glyceryl trinitrate (ampoules containing 1 mg/ml in isotonic saline) and pentobarbital sodium (Sagatal) were obtained from Lipha Pharmaceuticals (London, UK) and May&Baker (Dagenham, Essex, UK), respectively. [^{3}H]-L-Arginine was purchased from Amersham International (Buckinghamshire, UK).

2.2. RbCC preparation

Male New Zealand white rabbits (2.0–2.5 kg, provided by CEMIB-UNICAMP) were anaesthetised with pentobarbital sodium (Sagatal®, 30–40 mg/kg, i.v.) and exsanguinated via the carotid artery. Following penectomy, the RbCC was carefully dissected in chilled Krebs solution and cleared of the tunica albuginea and the surrounding tissues. Strips of RbCC were superfused in a cascade system with warmed (37°C) and oxygenated (95%O_2 + 5%CO_2) Krebs solution at a flow rate of 5 ml/min (Vane, 1964). The tissue responses (tension of 2.5 g) were detected with auxotonic levers attached to Harvard heart/smooth muscle transducers and displayed on a Watanabe multichannel pen recorder (model WTR 381). After a 60 min period of equilibration, RbCC strips were precontracted with noradrenaline (3 μM) in order to increase the basal tone. *A. australis* and *B. judaicus* venoms were administered as single bolus injections (10–100 μl). Indomethacin was continuously infused over tissues to prevent the generation of cyclo-oxygenase products.

2.3. Determination of rabbit cerebellar nitric oxide synthase (NOS) activity in vitro

The in vitro action of N^{ω}-nitro-L-arginine methyl ester (L-NAME), D-NAME and 7-NI was studied in rabbit cerebellum homogenates by measuring their ability to inhibit the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, as previously described (Förstermann et al., 1990). Briefly, the rabbits were anaesthetised with Sagatal (40 mg/kg, i.v.), the cerebella were rapidly removed and homogenised in five volumes of cold incubation buffer (50 mM Tris-HCl buffer, pH 7.4) containing 1 mM PMSF and 1 mM L-citrulline. The homogenates were incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$ and 10 mM L-arginine containing 100,000 dpm of [2,3,4,5-^{3}H] L-arginine monohydrochloride at room temperature (25–27°C). The NOS inhibitors L-NAME (and its inactive enantiomer D-NAME) or 7-NI were then added to the homogenates to give a final concentration of 0.01–3000 μM. The protein content of the samples was determined according to the method of Peterson (1977), and the activity of cerebellar NOS was expressed as pmol of L-citrulline/min/mg of protein. The reduction in L-citrulline production caused by the inhibitors was expressed as a percentage of the maximum activity. From the semi-log concentration–activity curves, the values of pI_2 ($-\log_{10}$ of the molar concentration of inhibitor that causes 50% inhibition) were calculated.

2.4. Data analysis

The relaxations induced by AAV, BJV and acetylcholine were measured considering the maximal relaxation induced by glyceryl trinitrate (dose in mol) as 100%. Results are expressed as means ± SEM of n experiments. Analysis of variance (ANOVA) and Student's unpaired t-test were employed to evaluate the data. $p < 0.05$ was taken as significant.

3. Results

3.1. Mediation by NO of the RbCC relaxations induced by the scorpion venoms

Bolus injection of AAV or BJV over the RbCC tissues caused dose-dependent relaxations (AAV: 16 ± 4, 47 ± 6 and 82 ± 7% relaxation; BJV: 7 ± 2, 23 ± 4 and 53 ± 6% relaxation, for 3, 10 and 30 μg, respectively; $n = 8$). Dose-dependent RbCC relaxations were also observed with acetylcholine (ACh; 65 ± 6, 79 ± 7 and 93 ± 10% for 0.2, 0.6 and 2 nmol, respectively; $n = 8$) and glyceryltrinitrate (GTN; 48 ± 5, 100 and 135 ± 13% for 0.5, 1.5 and 4.5 nmol, respectively, $n = 8$).

Fig. 1 shows that the infusion of D-NAME (10 μM; $n = 6$) affected neither the basal tone of the RbCC tissues nor the relaxations induced by ACh (0.6 nmol), AAV (30 μg) and BJV (30 μg). The subsequent infusion of NO synthase inhibitor L-NAME (10 μM; $n = 8$) further increased the tone of the RbCC tissues and markedly reduced ($p < 0.01$) the relaxations elicited by ACh, AAV and BJV without affecting those induced by GTN (1.5 nmol; Table 1). Subsequent infusion of L-arginine (300 μM; $n = 8$), but not of D-arginine (300 μM; $n = 6$), partially reversed the increased tone and significantly

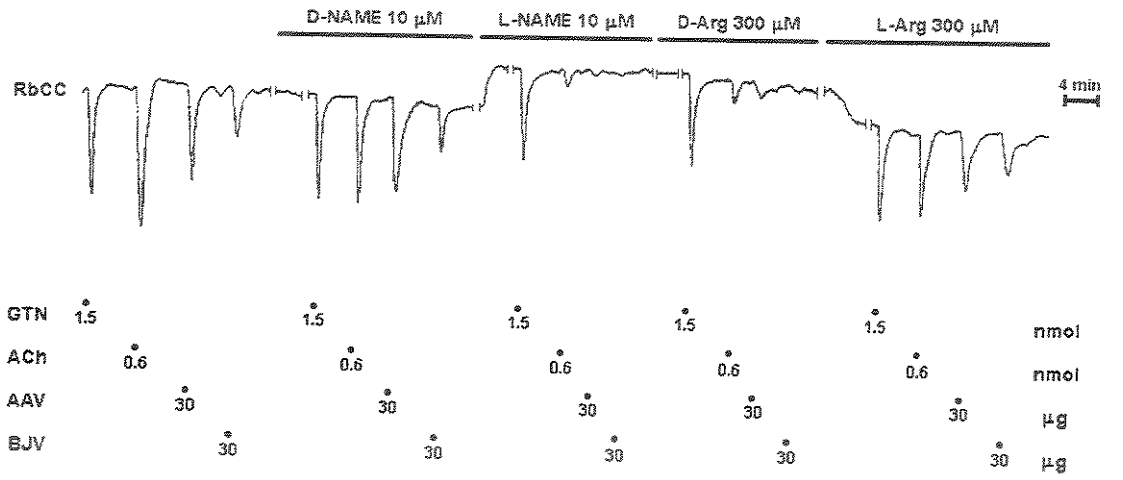

Fig. 1. *Androctonus australis* venom and *Buthotus judaicus* venom relax the rabbit corpus cavernosum (RbCC) due to NO release. The infusion of L-NAME (10 μM), but not D-NAME (10 μM), increased the RbCC tone and markedly reduced the relaxations induced by acetylcholine (ACh, 0.6 nmol), *Androctonus australis* venom (AAV, 30 μg) and *Buthotus judaicus* venom (BJV, 30 μg). The relaxations induced by glyceryl trinitrate (GTN, 1.5 nmol) were not significantly affected by L-NAME. Subsequent infusion of L-arg (300 μM), but not D-arg (300 μM), partially reversed the increased RbCC tone and also significantly restored the relaxations induced by ACh, AAV and BJV. This is a representative tracing of eight experiments.

restored ($p < 0.01$) the relaxations induced by ACh, AAV and BJV (Table 1).

The infusion of the neuronal NO synthase (nNOS) inhibitor 7-nitroindazole (7-NI; 10 μM, $n = 6$) increased the tone of the RbCC tissues (not shown) and markedly reduced the RbCC relaxation induced by both AAV (30 μg; 76 ± 11% before and 15 ± 3% during 7-NI infusion; $p < 0.01$) and BJV (30 μg; 85 ± 9% before and 33 ± 10% during 7-NI infusion; $p < 0.01$). In addition, 7-NI significantly inhibited the ACh-induced relaxations (0.6 nmol; 91 ± 13% before and 47 ± 11% during 7-NI infusion; $p < 0.05$). The inhibition of RbCC relaxations by 7-NI was reversible, since after stopping 7-NI infusion, relaxations could be fully restored (not shown). The GTN (1.5 nmol)-induced relaxations were not affected by 7-NI.

The infusion of 1H-[1,2,4] oxadiazolo [4,3,-alquinoxalin-1-one] (ODQ, 10 μM; $n = 5$), a selective inhibitor of NO-stimulated soluble guanylate cyclase activity, abolished ($p < 0.01$) the RbCC relaxations induced by ACh (0.6 nmol), AAV (30 μg) and BJV (30 μg; Table 2). The GTN (1.5 nmol)-induced RbCC relaxation was also significantly reduced by ODQ (97 ± 1% inhibition; $p < 0.01$). The relaxations evoked by these agents were significantly restored 25 min after stopping the ODQ

Table 1
Effect of D-NAME, L-NAME, D-arginine (D-arg) and L-arginine (L-arg) on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh), *Androctonus australis* venom (AAV) and *Buthotus judaicus* venom (BJV). D-NAME (10 μM), L-NAME (10 μM), D-arg (300 μM) and L-arg (300 μM) were infused over the RbCC tissues at a flow rate of 0.1 ml/min for at least 20 min before injection of the substances mentioned above. The RbCC relaxations induced by ACh, AAV and BJV were expressed (mean ± SEM, $n = 6$–8) relative to the sub-maximal relaxation induced by glyceryl trinitrate which was taken to be 100% (*$p < 0.01$ compared to the respective control)

Treatment	RbCC relaxations (%)					
	ACh (0.6 nmol)		AAV (30 μg)		BJV (30 μg)	
	Control	Treated	Control	Treated	Control	Treated
D-NAME	136 ± 11	121 ± 10	77 ± 8	66 ± 6	50 ± 2	47 ± 2
L-NAME	121 ± 10	24 ± 13*	66 ± 6	8 ± 2*	47 ± 2	0*
D-ARG	24 ± 13	23 ± 14	8 ± 2	3 ± 2	0	1 ± 1
L-ARG	23 ± 14	92 ± 4*	3 ± 2	48 ± 7*	1 ± 1	32 ± 4*

Table 2
Effect of ODQ and methylene blue (MB) on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh), *Androctonus australis* venom (AAV) and *Buthotus judaicus* venom (BJV). ODQ (10 μM) and MB (30 μM) were infused over the RbCC tissues at a flow rate of 0.1 ml/min for 20 min before injection of the substances mentioned above. The RbCC relaxations induced by ACh, AAV and BJV were expressed (mean ± SEM, $n = 5$) relative to the sub-maximal relaxation induced by glyceryl trinitrate which was taken to be 100% (*$p < 0.01$ compared to the respective control)

Treatment	RbCC relaxations (%)					
	ACh (0.6 nmol)		AAV (30 μg)		BJV (30 μg)	
	Control	Treated	Control	Treated	Control	Treated
ODQ	80 ± 15	1 ± 1*	94 ± 7	2 ± 1*	68 ± 5	1 ± 1*
MB	127 ± 14	35 ± 4*	112 ± 11	6 ± 1*	75 ± 7	2 ± 2*

infusion ($p < 0.01$; Fig. 2). The infusion of methylene blue (MB; 30 μM, $n = 4$) significantly reduced ($p < 0.01$) RbCC relaxations induced by ACh (0.6 nmol), AAV (30 μg) and BJV (30 μg; Table 2). The GTN (1.5 nmol)-induced RbCC relaxation was also significantly reduced by methylene blue (44 ± 11% inhibition; $p < 0.05$). However, in contrast to ODQ, the inhibitory action of methylene blue was irreversible (not shown).

3.2. Lack of effect of muscarinic receptor antagonists

Infusion of either atropine (1 μM; $n = 5$) or scopolamine (1 μM; $n = 5$) significantly reduced the ACh (0.6 nmol)-induced RbCC relaxation ($p < 0.01$), but had no significant effect on those induced by AAV (30 μg) or BJV (30 μg; Table 3).

3.3. Involvement of Na^+ channels

The infusion of the Na^+ channel blocker tetrodotoxin (TTX, 1 μM; $n = 6$) virtually abolished the RbCC relaxations elicited by both AAV (30 μg; 62 ± 16% before and 9 ± 4% during TTX infusion; $p < 0.01$) and BJV (30 μg; 27 ± 7% before and 1 ± 1% during TTX infusion; $p < 0.01$). At this concentration, TTX affected neither the tone of the RbCC tissues nor the relaxations induced by ACh (0.6 nmol; 138 ± 17% before and 119 ± 15% during TTX infusion) or GTN (1.5 nmol). A representative tracing is shown in Fig. 3.

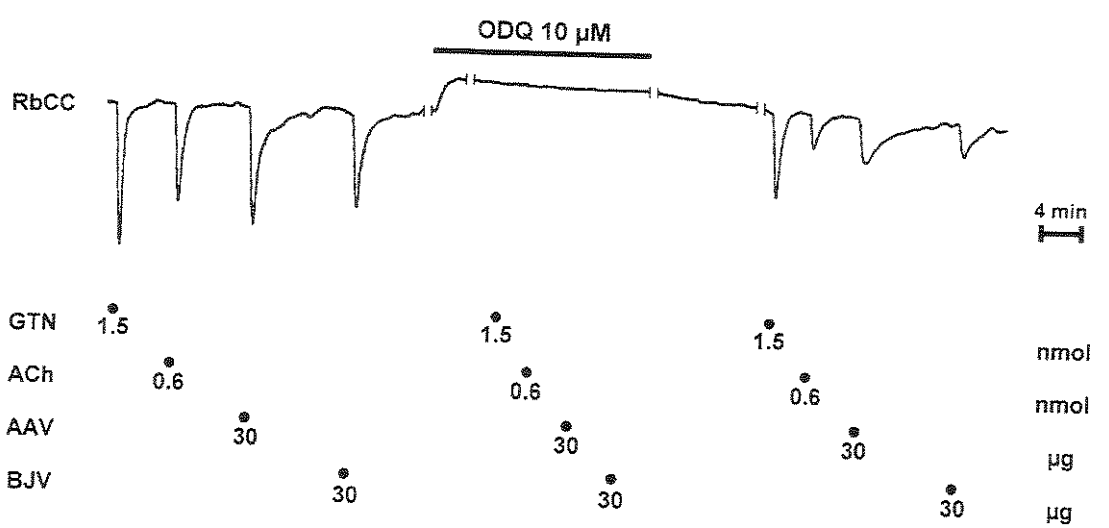

Fig. 2. Effects of ODQ on the relaxations elicited by scorpion venom. ODQ (10 μM) increased the tone of the rabbit corpus cavernosum (RbCC) strips and virtually abolished the relaxations induced by *Androctonus australis* venom (AAV, 30 μg), *Buthotus judaicus* venom (BJV, 30 μg), acetylcholine (ACh, 0.6 nmol) and glyceryl trinitrate (GTN, 1.5 nmol). After stopping ODQ infusion, the relaxations induced by these agents were partially restored. This is a representative tracing of five experiments.

Table 3
Effect of scopolamine and atropine on the rabbit corpus cavernosum (RbCC) relaxations induced by acetylcholine (ACh), *Androctonus australis* venom (AAV) and *Buthotus judaicus* venom (BJV). Scopolamine (1 μM) and atropine (1 μM) were infused over the RbCC tissues at a flow rate of 0.1 ml/min for 20 min before injection of the substances mentioned above. The RbCC relaxations induced by ACh, AAV and BJV were expressed (mean ± SEM, $n = 5$) relative to the sub-maximal relaxation induced by glyceryl trinitrate which was taken to be 100% (*$p < 0.01$ compared to the respective control)

Treatment	RbCC relaxations (%)					
	ACh (0.6 nmol)		AAV (30 μg)		BJV (30 μg)	
	Control	Treated	Control	Treated	Control	Treated
Scopolamine	103 ± 8	5 ± 1*	61 ± 5	66 ± 5	52 ± 5	59 ± 5
Atropine	119 ± 20	3 ± 1*	62 ± 9	81 ± 9	50 ± 5	66 ± 7

3.4. Effect of L-NAME, D-NAME and 7-NI on the activity of rabbit cerebellar NOS

The maximum NOS activity in the rabbit homogenates (in the absence of any inhibitor) was 3.61 ± 0.67 pmol L-citrulline/min/mg protein ($n = 4$). When Ca^{2+} was omitted from the incubation media, the conversion of L-arginine to L-citrulline was inhibited more than 90%, thus confirming that the measured enzymatic activity in the homogenates was due to a calcium-dependent NOS. From the concentration vs. NOS activity curves, the derived pI_2 values were 3.39 ± 0.62, 5.15 ± 0.26 and 5.02 ± 0.41 for D-NAME, L-NAME and 7-NI, respectively.

4. Discussion

The present study showed that *A. australis* and *B. judaicus* scorpion venoms cause NO-dependent RbCC relaxations. This conclusion was based on our findings that the non-selective NO synthase inhibitor L-NAME (but not its inactive enantiomer D-NAME) significantly reduced the

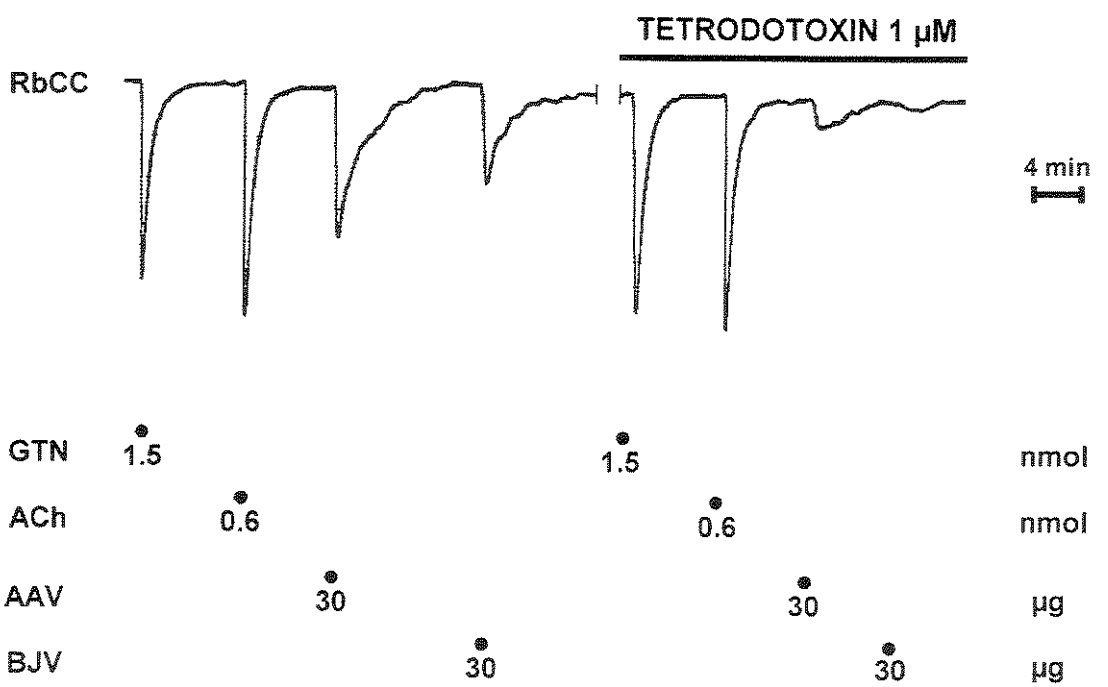

Fig. 3. The effect of the Na^+ channel blocker tetrodotoxin on the scorpion venom-induced rabbit corpus cavernosum (RbCC) relaxations. Tetrodotoxin (1 μM) virtually abolished the relaxations induced by *Androctonus australis* venom (AAV, 30 μg) and *Buthotus judaicus* venom (BJV, 30 μg), without affecting those elicited by either acetylcholine (ACh, 0.6 nmol) or glyceryl trinitrate (GTN, 1.5 nmol). This is a representative tracing of six experiments.

venom-induced relaxations. The reduction by L-NAME was partially reversed by the NO precursor L-arginine (but not D-arginine), thus confirming the involvement of NO in the relaxant responses elicited by both AAV and BJV.

NO has been identified as an important inhibitory neurotransmitter of NANC relaxations in the penile corpus cavernosum of man (Kim et al., 1991; Bush et al., 1992; Holmquist et al., 1992), rabbit (Ignarro et al., 1990; Kim et al., 1991; Bush et al., 1992; Holmquist et al., 1992), dog (Hedlund et al., 1995; Hayashida et al., 1996), horse (Recio et al., 1998), monkey (Okamura et al., 1998) and mouse (Göçmen et al., 1997). NO and vasodilators acting through NO release such as GTN (Feelisch, 1991) stimulate soluble guanylyl cyclase, leading to an increase in the tissue levels of cyclic GMP (Ignarro et al., 1990; Bush et al., 1992; Holmquist et al., 1993). The guanylyl cyclase inhibitors methylene blue (Gruetter et al., 1981; Rapoport and Murad, 1983) and ODQ (Garthwaite et al., 1995) markedly reduced the relaxations induced by either AAV or BJV, providing further evidence that venom-induced relaxations involves NO release which in turn activates the soluble guanylyl cyclase leading to cyclic GMP accumulation in the cavernosal smooth muscle.

Acetylcholine has been shown to present both pre- and post-junctional inhibitory effects on adrenergic neurotransmission in isolated penile erectile tissue (Hedlund and Andersson, 1985). The failure of the muscarinic receptor antagonists atropine and scopolamine to reduce the RbCC relaxations elicited by AAV and BJV suggests that acetylcholine release does not contribute to the venom-induced relaxations and excludes the involvement of cholinergic nerves in the venom transduction mechanism of action. Accordingly, muscarinic receptor blockade has no effect on the rat (Dail et al., 1987) and rabbit (Holmquist et al., 1992) corpus cavernosum relaxations elicited by electrical field stimulation, thus suggesting a role for NO supplied by NANC nitrergic terminals as the main neural mediator in penile erection.

In our study, the relaxant responses of rabbit cavernosal smooth muscle preparations to scorpion venoms were susceptible to full blockade by the Na^+ channel blocker tetrodotoxin, indicating that they originated from the stimulation of nerves supplying the cavernosal tissue. Furthermore, the presence of NO synthase has been demonstrated histochemically in neurones in the rat, dog and human penis (Burnett et al., 1992; Keast, 1992; Burnett et al., 1993). The compound 7-NI, an inhibitor of nNOS (Moore et al., 1993), has been reported to induce a reversible and dose-dependent inhibition of erection in the rat (Spiess et al., 1996). Our findings that 7-NI markedly reduced the venom-induced relaxations in a reversible fashion suggest that NO released by AAV and BJV in the rabbit cavernosal tissue is neuronal in origin. Besides preferentially inhibiting the nNOS, 7-NI has also been shown to inhibit the endothelial NO synthase (eNOS) isoform in vitro (Wolff et al., 1994; Bland-Ward and Moore, 1995). This may explain the ability of 7-NI to increase the basal RbCC tone and to reduce acetylcholine-induced relaxations. Additionally, using rabbit cerebellum homogenates we demonstrated that 7-NI and L-NAME were equipotent regarding the inhibition of nNOS.

On the basis of these findings we conclude that both AAV and BJV relax the cavernosal tissues by activating NANC nerve fibres with subsequent release of NO that in turn leads to relaxation through activation of soluble guanylate cyclase and hence cyclic GMP formation.

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Biochemical Pharmacology

Role of nitric oxide on the increased vascular permeability and neutrophil accumulation induced by staphylococcal enterotoxin B into the mouse paw

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Abstract

The role of nitric oxide (NO) on the increase in vascular permeability and neutrophil migration induced by staphylococcal enterotoxin B (SEB; 25 μg/paw) in the mouse was investigated in this study. The NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) [but not its inactive enantiomer N^{ω}-nitro-D-arginine methyl ester (D-NAME)], given intravenously (25–100 μmol/kg) or subplantarly (0.25–1.0 μmol/paw), reduced SEB-induced paw oedema significantly. A similar response was observed with aminoguanidine, given either intravenously (200–600 μmol/kg) or subplantarly (2 μmol/paw). In contrast to paw oedema, the plasma exudation in response to SEB was not affected by the subplantar injection of L-NAME or aminoguanidine. The inhibition of oedema and plasma exudation by systemic treatment with L-NAME or aminoguanidine was reversed by co-injection of the vasodilator iloprost (0.3 nmol/paw). Subplantar injection of SEB (25 μg/paw) increased by 69% the myeloperoxidase (MPO) activity of SEB-treated paws, indicating the presence of neutrophils. Intravenous (12.5–50 μmol/kg) or subplantar (0.125–0.5 μmol/paw) administration of L-NAME (but not of its inactive enantiomer, D-NAME) largely reduced the MPO activity in SEB-treated paws. Similarly, intravenous (200–600 μmol/kg) or subplantar (2 μmol/paw) administration of aminoguanidine significantly reduced the MPO values of the SEB-injected paws. The vasodilator iloprost (0.3 nmol/paw) completely reversed the inhibition by L-NAME or aminoguanidine of the MPO activity in SEB-injected paws. Our results show that the increased vascular permeability and neutrophil accumulation in response to subplantar injection of SEB in the mouse are inhibited by L-NAME and aminoguanidine by mechanisms probably involving reduction of local microvascular blood flow.

Keywords: Plasma exudation; Paw oedema; Neutrophils; Enterotoxins; Aminoguanidine

1. Introduction

Staphylococcal enterotoxins are the most common cause of acute food poisoning in humans [1]. They represent a family of structurally related exoproteins (25–30 kDa) produced by several strains of *Staphylococcus aureus* and are classified into eight distinct immunological types (A-E and G-I) [2]. The physiopathological responses to staphylococcal enterotoxins involve activation of a number of cell types such as mast cells, macrophages, and lymphocytes, causing the release of different mediators including histamine, serotonin, leukotrienes, and various cytokines [3–5]. Among the staphylococcal toxins, SEB can be easily obtained in relatively large amounts and purity, and therefore has been used extensively in experimental investigations. Previous studies demonstrated that SEB induces hind-paw oedema and peritoneal neutrophil migration in the mouse, both of which are believed to be mediated by platelet-activating factor, histamine, leukotrienes, and neuropeptides [6,7]. In addition, SEB causes acute inflammatory lung injury characterized by an increase in vascular permeability, granulocyte infiltration, and induction of a number of cell adhesion

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Abbreviations: SEB, staphylococcal enterotoxin B; NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; D-NAME, N^{ω}-nitro-D-arginine methyl ester; MPO, myeloperoxidase; IL, interleukin; TNF, tumor necrosis factor; and IFN, interferon.

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molecules [8]. In the air pouch model in mice, SEB was able to recruit leukocytes (mainly neutrophils) in the pouch exudate [9]. Neutrophils have been considered the major cell type involved in the host's defense against *S. aureus* [10], and accumulation of neutrophils is a prominent feature of staphylococcal enterotoxin-induced gastroenteritis [11].

NO is produced in physiopathological conditions by three distinct isoforms of NOS, namely eNOS (isoform III), bNOS (isoform I), and iNOS (isoform II). The first two isoforms are expressed constitutively, whereas iNOS is expressed following exposure to diverse stimuli, such as cytokines (IL-1, TNF, IFN-γ) and lipopolysaccharide. SEB is able to release NO both *in vivo* [12] and *in vitro* [13], and this seems to play a major role in SEB-induced shock. Since NO has been shown to modulate acute and chronic inflammatory processes [14], in this study we examined the role of NO on the increase in vascular permeability (plasma exudation and paw oedema) and neutrophil migration induced by SEB in the mouse paw.

2. Materials and methods

2.1. Drugs

SEB (purchased from the Sigma Chemical Co.) was dissolved in sterile saline (0.9%, w/v) and stored in a refrigerator at a concentration of 1 mg/mL. L-NAME, D-NAME, aminoguanidine, hexadecyltrimethylammonium (HTAB), *o*-dianisidine dihydrochloride, and hydrogen peroxide were also obtained from Sigma. Iloprost was obtained from Schering. L-[2,3,4,5-^{3}H]Arginine (specific activity 60.0 Ci/mmol) was supplied by Amersham. All drugs were diluted in saline.

2.2. Animals

All experiments were carried out in accordance with the guidelines of the State University of Campinas (UNICAMP). Experimental procedures were performed in adult male Swiss mice (25–30 g) obtained from the Central Animal House (CEMIB).

2.3. Measurement of paw oedema and vascular permeability

Groups of five mice were injected intravenously with Evans blue (25 mg/kg) immediately before subplantar injection of 0.05 mL (25 μg/paw) of SEB in the left hind-paw under light ether anesthesia. The right hind-paw of each mouse served as the control and received an injection of 0.05 mL of vehicle alone (saline). The oedema and the dye exudate were measured 4 hr after the stimulus. Briefly, after being killed, the paws of the animals were amputated at the tarsocrural joint and weighed on an analytical balance. The oedema, expressed in milligrams, was the weight variation between the left (treated) and right (untreated) paw. Each paw was then chopped into small pieces and placed in a test tube with 3.0 mL of formamide. The tubes were then incubated in a water bath at 57° for 24 hr. The absorbance of the supernatants was measured at 619 nm. The concentration of Evans blue present in the extracts was determined from a standard curve of the dye prepared in formamide.

2.4. MPO assay

MPO, a hemoprotein located in azurophil granules of neutrophils, has been used as a biochemical marker for neutrophil infiltration into tissues. Briefly, each paw was weighed, chopped into small pieces, and placed in a test tube in the presence of 0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0. Each tissue sample was homogenized for 15 sec, then vortexed, and 1-mL aliquots of the homogenate were decanted into Eppendorf tubes. These tubes were then centrifuged (Eppendorf centrifuge) for 2 min at maximum speed, and the supernatants were collected. An MPO assay was performed using a microliter plate scanner (Spectra Max 34, Molecular Devices). This consisted of mixing 10 μL of sample with 200 μL of *o*-dianisidine solution (0.167 mg/mL of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide) prior to reading the plate. The changes in absorbance were measured at 460 nm for 30 sec over a period of 5 min. One unit of MPO activity was defined as that degrading 1 μmol of peroxide/min at 25° [15].

2.5. NOS assay

NOS activity was determined according to the method described by Förstermann *et al.* [16], which is based upon the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Briefly, hind-paws were thawed, weighed, and homogenized with 50 mM Tris–HCl, pH 7.4, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM L-citrulline in a polytron tissue homogenizer. The homogenates were centrifuged (10,000 *g*, 10 min), and 50 μL of each supernatant was incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$, 10 μg/mL of calmodulin, 10 μM FAD, 100 μM BH_4, and 10 μM L-arginine containing 200,000 dpm of [2,3,4,5-^{3}H]L-arginine monohydrochloride at 37°. Pharmacological controls of NOS activity were carried out in parallel and consisted of either the omission of $CaCl_2$ and the addition of 1 mM EGTA, or the addition of 1 mM L-NAME to the incubation medium. The protein content of samples was determined according to the method of Peterson [17], and NOS activity was expressed as picomoles of L-citrulline per minute per milligram of protein.

2.6. Experimental protocols

For the plasma exudation and paw oedema measurements, the animals received L-NAME (25–100 μmol/kg),

D-NAME (100 μmol/kg), or aminoguanidine (200–600 μmol/kg) intravenously immediately before subplantar injection of SEB (25 μg/paw; N = 5 in each group). In a separate set of experiments, L-NAME (0.25–1.0 μmol/paw), D-NAME (1.0 μmol/paw), and aminoguanidine (2 μmol/paw) were co-injected with SEB into the paws (N = 5 in each group). In the experiments designed to examine the effects of the vasodilator iloprost on SEB-induced responses, iloprost (0.3 nmol/paw) was injected 1 hr before the measurement of paw oedema. The increases in paw weight and plasma exudation were always measured 4 hr after the subplantar injection of SEB.

For the MPO assay, groups of five mice were injected intravenously with L-NAME (12.5–50 μmol/kg), D-NAME (50 μmol/kg), or aminoguanidine (200–600 μmol/kg) immediately before the subplantar injection of SEB (25 μg/paw). In a separate set of experiments, L-NAME (0.125–0.5 μmol/paw), D-NAME (0.5 μmol/paw), and aminoguanidine (2 μmol/paw) were co-injected with SEB into the paws (N = 5 in each group). In the experiments designed to examine the effects of the vasodilator iloprost on SEB-induced responses, iloprost (0.3 nmol/paw) was injected 1 hr before the measurement of MPO activity. The MPO activity in the paw homogenates was determined 4 hr after the subplantar injection of SEB.

2.7. Statistical analysis

Data are reported as the means ± SEM of 4–5 animals. Results were compared using ANOVA followed by the Bonferroni test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Plasma exudation and paw oedema

Subplantar injection of SEB in the hind-paws of mice caused a dose-dependent paw oedema (2 ± 2, 10 ± 0.1, 30 ± 3.1, and 32 ± 2 mg for 6.25, 12.5, 25, and 50 μg/paw at 4 hr, respectively) and plasma exudation (18 ± 2, 32 ± 2, and 14 ± 2.5 mg for 25 μg/paw at 2, 4, and 24 hr, respectively). The time-course for oedema formation (18 ± 2, 32 ± 2, and 14 ± 2.5 mg for 25 μg/paw at 2, 4, and 24 hr, respectively) and plasma exudation (1.4 ± 0.4, 4.3 ± 0.6, and 1.8 ± 0.2 μg/g of Evans blue for 25 μg/paw at 2, 4, and 24 hr, respectively; N = 5) revealed that maximal responses were observed at 4 hr post-SEB injection. For further studies, SEB was administered routinely at a dose of 25 μg/paw, and both plasma protein exudation and paw oedema were evaluated 4 hr later.

Figure 1A shows that intravenous administration of L-NAME (25–100 μmol/kg) caused a dose-dependent inhibition of the SEB-induced paw oedema. A significant reduction ($P < 0.05$) in dye exudation was observed only at the highest dose of L-NAME (Fig. 1B). The intravenous administration of the inactive enantiomer D-NAME (100 μmol/kg) affected neither paw oedema (Fig. 1A) nor plasma exudation (Fig. 1B). At the doses used, intravenous administration of L-NAME had no significant effect on basal plasma exudation in saline-injected paws (not shown).

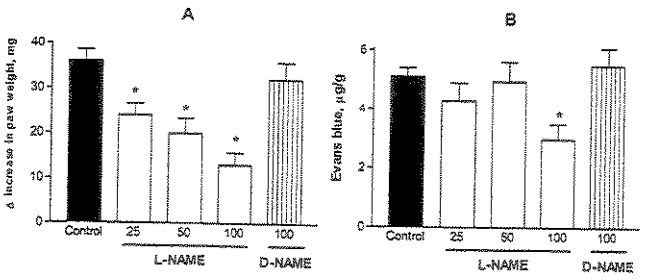

Fig. 1. Effects of L-NAME (25–100 μmol/kg) and D-NAME (100 μmol/kg) on SEB-induced paw oedema and plasma exudation. The changes in paw weight (A) and Evans blue exudation (B) were measured 4 hr after the injection of SEB (25 μg/paw). Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group (untreated animals).

The subplantar administration of L-NAME (0.25–1.0 μmol/paw), but not D-NAME (1.0 μmol/paw), also reduced SEB-induced paw oedema significantly without affecting plasma exudation (Table 1). At the doses used, subplantar administration of L-NAME had no significant effect on the basal plasma exudation of the saline-injected paws (not shown).

The intravenous administration of the iNOS inhibitor aminoguanidine (200–600 μmol/kg) significantly reduced ($P < 0.05$) both paw oedema (Fig. 2A) and plasma exudation (Fig. 2B) induced by SEB. When given locally into the paws, aminoguanidine (2 μmol/paw) reduced paw oedema by 44% ($P < 0.05$), but failed to affect plasma exudation (4.6 ± 0.8 and 5.6 ± 0.5 μg/g of Evans blue for control and treated-paws, respectively). At the doses used, subplantarly or intravenously, aminoguanidine had no significant effect on basal plasma exudation in saline-injected paws (not shown).

Table 1
Effects of subplantar administration of L-NAME or D-NAME on paw oedema and plasma exudation induced by SEB (25 μg/paw)

	Δ Increase in paw weight (mg)	Evans blue (μg/g)
Control	34 ± 2.4	5.0 ± 0.3
L-NAME (μmol/paw)		
0.25	18 ± 2.0*	4.6 ± 1.1
0.5	20 ± 3.2*	4.7 ± 0.9
1.0	20 ± 4.5*	4.8 ± 0.6
D-NAME (μmol/paw)		
1.0	30 ± 3.2	4.9 ± 0.9

The increase in paw weight and Evans blue exudation was measured 4 hr after SEB injection. Data represent the means ± SEM of five mice.
*$P < 0.05$ compared with the control group.

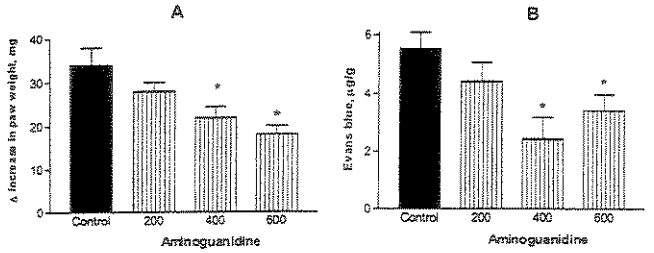

Fig. 2. Effect of aminoguanidine (200–600 μmol/kg, i.v.) on SEB-induced paw oedema and plasma exudation. The changes in paw weight (A) and Evans blue exudation (B) were measured 4 hr after injection of SEB (25 μg/paw). Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group (untreated animals).

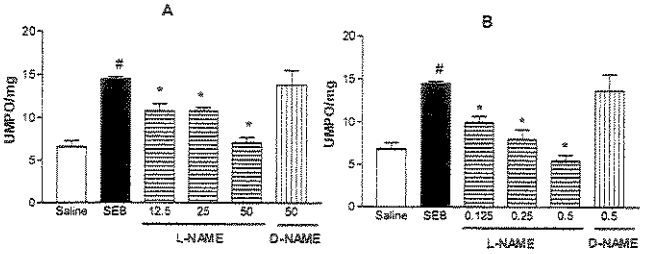

Fig. 4. Effects of intravenous (A) and subplantar (B) administration of L-NAME and D-NAME on the MPO activity of SEB-treated paws. The measurement of MPO activity (UMPO/mg) was carried out at 4 hr after injection of SEB (25 μg/paw). UMPO = MOP units. Each column represents the mean ± SEM of five mice. Key: (#) $P < 0.05$ compared with the saline group; and (*) $P < 0.05$ compared with the SEB group.

The subplantar injection of the prostacyclin analogue iloprost (0.3 nmol/paw) potentiated the SEB-induced paw oedema and plasma exudation by 65.7 and 42.7% ($P < 0.05$), respectively, as expected. In addition, iloprost significantly reversed the inhibition by L-NAME or aminoguanidine of the SEB-induced paw oedema (Fig. 3A) and plasma exudation (Fig. 3B). Iloprost did not evoke paw oedema or plasma exudation when injected alone (not shown).

3.2. Measurement of MPO activity

Subplantar injection of SEB (25 μg/paw) in the hindpaws of the mice caused a significant increase ($P < 0.05$) in MPO activity, as determined at 4 and 12 hr post-SEB injection (12.5 ± 0.8 and 15.7 ± 1.1 MPO units/mg) in comparison with the MPO values in the contralateral paws injected with sterile saline (7.44 ± 0.5 MPO units/mg). For further studies, MPO activity was determined routinely at 4 hr post-SEB injection.

Intravenous administration of L-NAME (12.5–50 μmol/kg) largely reduced the MPO activity in SEB-treated paws. At the highest dose (50 μmol/kg), the increased MPO activity was nearly abolished by L-NAME (Fig. 4A). Similarly, subplantar administration of L-NAME (0.125–0.5 μmol/paw) dose-dependently reduced the increased MPO activity in SEB-treated paws. A total reversal of the increased MPO activity was observed with 0.5 μmol/paw of L-NAME (Fig. 4B). The inactive enantiomer D-NAME, given either intravenously (50 μmol/kg) or subplantarly (0.5 μmol/paw), had no effect on the increased MPO activity in SEB-treated paws (Fig. 4). In addition, L-NAME, given either intravenously (50 μmol/kg) or subplantarly (0.5 μmol/paw), failed to affect the MPO activity of saline-injected paws significantly (not shown).

The intravenous (200–600 μmol/kg) or subplantar (2 μmol/paw) administration of the iNOS inhibitor aminoguanidine significantly reduced ($P < 0.05$) MPO activity in the SEB-treated paws. However, a complete reduction of MPO activity was not observed with the use of aminoguanidine (Table 2).

Subplantar injection of iloprost (0.3 nmol/paw) potentiated by 40% ($P < 0.05$) the MPO activity of SEB-treated paws. In addition, iloprost completely reversed the inhibition by L-NAME (50 μmol/kg, i.v.) or aminoguanidine (600

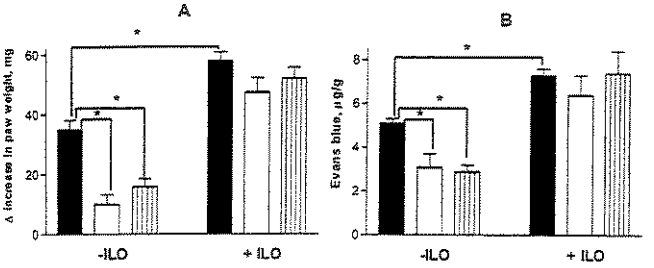

Fig. 3. Effects of iloprost on the inhibition of SEB-induced paw oedema (A) and plasma exudation (B) by L-NAME (open columns) or aminoguanidine (striped columns). The mice were injected intravenously with either L-NAME (100 μmol/kg) or aminoguanidine (600 μmol/kg) immediately before SEB (25 μg/paw). Control (untreated) mice were injected intravenously with saline (solid columns). The mice received either iloprost (0.3 nmol/paw; + ILO) or saline instead of iloprost (-ILO) 1 hr before paw oedema analysis. The changes in paw weight and dye exudation were measured 4 hr after the injection of SEB. Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group.

Table 2
Effects of intravenous (200–600 μmol-kg) or subplantar (2 μmol/paw) administration of aminoguanidine on MPO activity of SEB-treated paws (25 μg/paw)

	MPO activity (MPO units/mg)
Saline	6.9 ± 0.7
SEB	22.1 ± 1.5**
Aminoguanidine (μmol/kg)	
200	17.2 ± 0.6**
400	14.9 ± 1.0**
600	14.8 ± 1.1**
Aminoguanidine (μmol/paw)	
2	12.7 ± 1.2**

Changes in MPO activity were measured 4 hr after SEB injection. Data represent the means ± SEM of five mice.

* $P < 0.05$ compared with the saline group.

** $P < 0.05$ compared with the SEB-treated group.

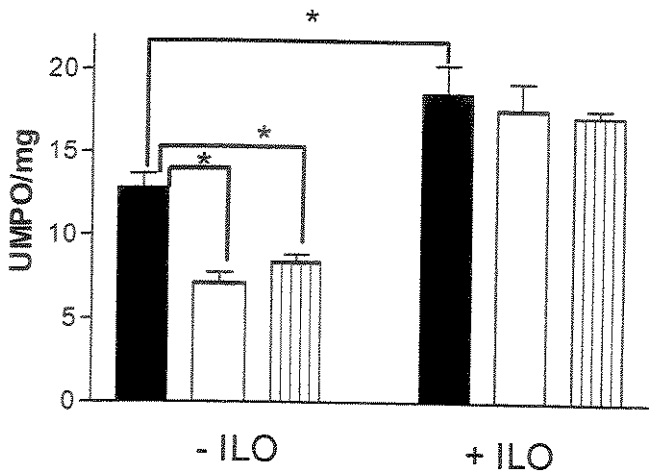

Fig. 5. Effects of iloprost on the reduction of MPO activity by L-NAME (open columns) or aminoguanidine (striped columns). The mice were injected intravenously with either L-NAME (50 μmol/kg) or aminoguanidine (600 μmol/kg) immediately before SEB (25 μg/paw). Control (untreated) mice were injected intravenously with saline (solid columns). The mice received either iloprost (0.3 nmol/paw; + ILO) or sterile saline instead of iloprost (-ILO). The changes in MPO activity (UMPO/mg) were measured 4 hr after the injection of SEB. UMPO = MPO units. Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group.

μmol/kg, i.v.) of the MPO activity in SEB-injected paws (Fig. 5). When injected alone, iloprost did not affect MPO activity compared with the saline control (7.5 ± 1.1 and 7.6 ± 0.4 MPO units/mg, respectively).

3.3. Measurement of NOS activity

cNOS activity in the SEB-injected mouse paws was enhanced markedly (1.7 ± 0.4 pmol citrulline/min/mg) compared with that of the saline-injected paws (0.4 ± 0.09 pmol citrulline/min/mg, N = 3; $P < 0.05$). The omission of Ca^{2+} and the addition of EGTA to the paw homogenates abolished the increased NOS activity of SEB-injected paws (0.3 ± 0.1 pmol citrulline/min/mg), thus indicating that the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was essentially due to constitutive NOS.

4. Discussion

Our results clearly show that increased vascular permeability and neutrophil accumulation in response to SEB in the mouse paw were inhibited significantly by local or systemic treatment of the animals with the NOS inhibitors L-NAME and aminoguanidine, strongly indicating that NO modulates both of these phenomena. The failure of the inactive enantiomer D-NAME to affect the SEB-induced inflammatory responses confirms the hypothesis that inhibition by L-NAME was, in fact, due to the inhibition of NO synthesis.

The role of NO in inflammation is still controversial. Depending on the type and the phase of inflammation and the individual vascular or cellular response studied, NO seems to have both pro-inflammatory and anti-inflammatory properties, suggesting that NO, like other inflammatory mediators, has a dualistic function in inflammation [14]. NO modulates vascular permeability and oedema formation by mechanisms dependent on, and independent of, local blood flow [18]. Our results showing that L-NAME, given intravenously or subplantarly, significantly reduced SEB-induced paw oedema are in agreement with previous studies that demonstrated that this compound reduces oedema formation in a number of animal species, including the mouse [19,20]. The reduction of inflammatory oedema by NOS inhibitors has been attributed to their ability to prevent vasodilatation and decrease regional microvascular blood flow as a consequence of the inhibition of NO synthesis in vascular endothelium [21–24]. We speculated, therefore, that inhibition of SEB-induced paw oedema by L-NAME reflects a decrease in blood flow of the paw microcirculation (vasoconstriction) without directly affecting the permeability. The finding that iloprost, an analogue of the vasodilator prostacyclin [25], completely reversed the inhibition by L-NAME of SEB-induced paw oedema is consistent with this proposal, as previously suggested for carrageenan-induced rat paw oedema [26]. Aminoguanidine is a compound known to preferentially inhibit the iNOS isoform with little or no inhibitory effect on the constitutive (endothelial and neuronal) NOS isoforms [27–29]. Interestingly, systemic or subplantar treatment of the mice with aminoguanidine also reduced SEB-induced paw oedema significantly, and such reduction was completely reversed by iloprost. This suggests that inhibition of SEB-induced oedema by aminoguanidine cannot be explained solely by virtue of its ability to selectively inhibit the iNOS isoform, but rather involves inhibition of constitutive NOS activity. Indeed, previous studies reported that, depending on the dose employed, aminoguanidine can inhibit constitutive NOS activity [30]. Our findings that subplantar injection of SEB markedly increased constitutive NOS activity in the paw homogenates further support the involvement of this isoform in oedema development. The participation of an inducible, calcium-independent NOS isoform was excluded since omission of calcium and addition of EGTA to the paw homogenates completely prevented the increased NOS activity. Although the evidence that SEB increases the activity of a constitutive (but not an inducible) NOS isoform is surprising, previous studies showed that rat paw oedema induced by carrageenan is accompanied by a marked increase in constitutive (but not inducible) NOS in paw homogenates at early (0–4 hr) phases of oedema [31], an effect recently attributed to NO synthesized by a neuronal NOS isoform, most probably within sensory nerves [32]. Accordingly, a recent study showed that SEB-induced mouse paw oedema is a consequence of a complex neurogenic response involving local activation of sensory nerves [33].

It is well documented that development of inflammatory oedema is due primarily to an increase in protein efflux, which decreases the lymph-to-plasma total-protein ratio (L/P ratio), thus virtually eliminating the transmural colloid osmotic pressure gradient, whereas the transmural hydrostatic pressure gradient is increased markedly. The rise in microvascular pressure (P_{mv}) is not accepted as an important determinant of oedema formation [34]. Our results showed that while L-NAME (or aminoguanidine) reduced SEB-induced paw oedema, the plasma exudation was reduced only by the higher doses of these compounds (in the case of systemic treatment) or was not affected (in the case of subplantar treatment). This implies that in situations where paw oedema is reduced, the amount of extravasated proteins is not modified significantly. Although these results are unclear, they suggest that L-NAME and aminoguanidine reduce SEB-induced paw oedema mainly by reducing the transmural hydrostatic pressure gradient; the findings that the vasodilator iloprost reversed the inhibition of paw oedema by L-NAME and aminoguanidine reinforce this proposal. Furthermore, L-NAME inhibits carrageenan-induced paw oedema without affecting the permeability kinetics [26]. In this respect, it is interesting to note that L-NAME has been shown to increase the microvascular protein efflux by mechanisms dependent on, and independent of, leukocyte adhesion [35,36]. However, it is unlikely that this mechanism contributes to the overall phenomenon in the mouse paw microcirculation since L-NAME and aminoguanidine failed to affect basal plasma exudation, as observed in the saline-treated paws.

Neutrophil migration from circulating blood to sites of injury is a crucial event during inflammatory processes, a phenomenon mediated by various substances including C5a, leukotriene B_4, IL-1, IL-8, TNF-α, and IFN-γ. Some of these cytokines such as IL-1, TNF-α, and IFN-γ can induce iNOS activity in a variety of cells (including neutrophils), leading to enhanced NO formation [30]. The role of NO on leukocyte recruitment has been investigated extensively, but results are still conflicting. Inhibitors of NOS such as N^G-monomethyl-L-arginine attenuated *in vitro* human neutrophil chemotaxis [37,38]. In the *in vivo* pleurisy model, L-NAME can either inhibit [39,40] or have no effect on [41] pleural neutrophil accumulation. Additionally, in both cat mesenteric [42] and rat hindlimb muscle [43] preparations *in vivo*, L-NAME increases leukocyte adhesion to vascular endothelium. Our results showed that subplantar injection of SEB markedly increased MPO activity in mouse paw homogenates at 4 hr, indicating that the number of neutrophils in the paws was enhanced by this enterotoxin. The increased MPO activity values of SEB-treated paws were reduced significantly by L-NAME (but not D-NAME) and aminoguanidine, indicating that NO modulates this phenomenon. Since L-NAME (and aminoguanidine) reduced SEB-induced paw oedema by a mechanism probably related to decrease of local blood flow, we next examined whether attenuation of infiltrating neutrophils by the NO inhibitors also reflects a decrease of regional microvascular blood flow, a condition that may restrict the number of infiltrating cells into inflammatory sites. Our findings that the vasodilator iloprost potentiated the MPO activity in SEB-treated paws and completely reversed the inhibitory effects of L-NAME (and aminoguanidine) on the SEB-induced increase in MPO activity are consistent with the suggestion that reduction of basal blood flow is the mechanism by which L-NAME and aminoguanidine inhibit neutrophil accumulation in SEB-treated paws. In a similar way, the vasodilators sodium nitroprusside and prostaglandin E_1 also reversed the inhibitory effects of L-NAME on the neutrophil accumulation induced by zymosan-activated serum in guinea-pig skin [23], thus reinforcing our proposal. Furthermore, the failure of L-NAME to affect the MPO activity in the contralateral paws injected with saline may rule out that increased MPO activity in SEB-treated paws reflects an enhancement of neutrophil adhesion to vascular endothelium.

In conclusion, our results demonstrate that the increased vascular permeability and neutrophil accumulation observed in response to subplantar injection of SEB in mice were inhibited significantly by NOS inhibitors such as L-NAME and aminoguanidine. Reduction of local blood flow seems to be the main mechanism by which the NO inhibitors exert their anti-inflammatory effects.

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Involvement of nitric oxide on the peritoneal neutrophil influx induced by staphylococcal enterotoxin B in mouse

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Abstract

In this study, the role of nitric oxide (NO) on neutrophil migration induced by staphylococcal enterotoxin B (SEB) in the mouse peritoneal cavity was investigated. The NO synthase inhibitors L-NAME and aminoguanidine, as well as dexamethasone, markedly reduced SEB-induced neutrophil influx. In mice with an increased population of peritoneal macrophages, the inhibition of SEB-induced neutrophil influx by these agents was significantly lower. The in vivo treatment with aminoguanidine inhibited only the iNOS activity, whereas L-NAME inhibited both the cNOS and iNOS activities. In conclusion, NO modulates the neutrophil migration in response to SEB through the activity of an iNOS isoform. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Enterotoxins; Aminoguanidine; Inflammation; Nitric oxide synthases

Staphylococcus aureus secretes several toxins known as staphylococcal enterotoxins (SE) which are a major cause of food poisoning in man. They represent a family of structurally-related exoproteins (25–30 kD) that are classified into eight distinct immunological types (Munson et al., 1998). The enterotoxin type B (SEB) causes acute inflammatory lung injury characterised by an increase in vascular permeability, granulocyte infiltration and induction of cell adhesion molecules (Neumann et al., 1997). Platelet-activating factor, histamine, leukotrienes (DeSouza et al., 1996; DeSouza and Ribeiro-DaSilva, 1996) and neuropeptides (Linardi et al., 2000) have been implicated in the inflammatory responses to SEB. In mice, intraplantar administration of SEB induces an early and long-lasting increase in vascular permeability and hence oedema formation (DeSouza et al., 1996) that is accompanied by a marked neutrophil influx into the paw tissues (Franco-Penteado et al., 2000). Nitric oxide (NO) modulates the SEB-induced inflammatory responses in the mice paw by a mechanism involving reduction of local microvascular blood flow through the activity of a constitutive NO synthase (NOS; Franco-Penteado et al., 2000). Additionally, SEB is able to potently attract neutrophils into the mice peritoneal cavity, the maximal response of which is obtained at 12 h post-toxin injection (DeSouza and Ribeiro-DaSilva, 1996). The plasma levels of nitrite and nitrate is also markedly increased at 16 h after intraperitoneal injection of SEB, thus indicating a marked in vivo NO synthesis (Florquin et al., 1994). Since NO has been shown to modulate the neutrophil chemotaxis (Kaplan et al., 1989; Belenky et al., 1993), this study was carried out to investigate the role of NO on the peritoneal neutrophil migration induced by SEB in mice.

The experiments were carried out in accordance with the guidelines of State University of Campinas (UNICAMP). The experimental procedures were performed in adult male Swiss mice (25–30 g) obtained from the Central Animal House. Staphylococcal enterotoxin B (Sigma, St Louis, USA, dissolved in sterile 0.9% PBS) was injected into the peritoneal cavity. Twelve hours thereafter, the cells from the peritoneal cavity were harvested by lavage of the cavities with 3 ml of PBS-heparin (5 IU/ml) prepared in 0.1% bovine serum albumin (BSA). Total and differential

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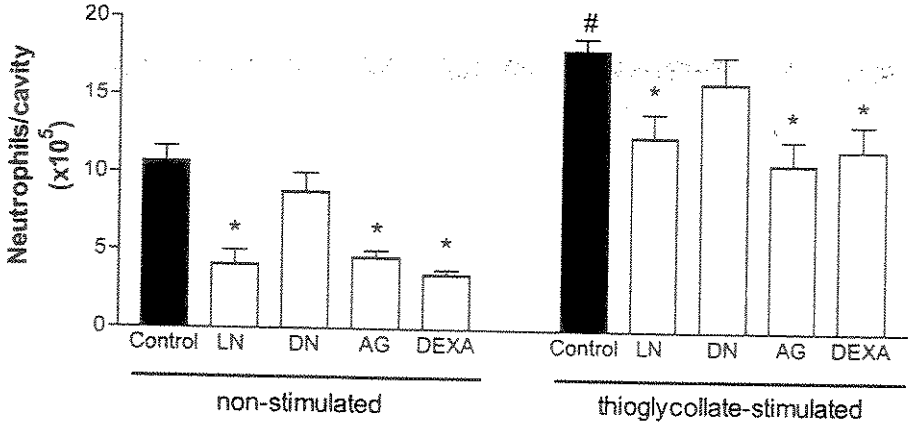

Fig. 1. The effect of L-NAME (LN; 100 μmol/kg), D-NAME (DN; 100 μmol/kg) aminoguanidine (AG; 400 μmol/kg) and dexamethasone (DEXA; 0.5 mg/kg) on the peritoneal neutrophil migration induced by SEB (125 μg/cavity) in both non-stimulated and thioglycollate-stimulated cavities. Neutrophil migration was measured 12 h after injection of SEB. Each column represents the mean ± SEM of five mice. $^{*}P < 0.05$ compared to respective control group; $^{\#}P < 0.05$ compared to control group of non-stimulated cavity.

cell counts were determined by conventional techniques. In a separate group of experiments, animals were pre-treated with 2 ml of thioglycollate (3% w/v, i.p.) to increase the number of peritoneal macrophages (Gallily and Feldman, 1967). Four days later, animals were injected with SEB (125 μg, i.p.) and neutrophil migration evaluated 12 h later. The effects of both L-NAME (St Louis, USA) and aminoguanidine (St Louis, USA) on the in vivo cNOS (brain) and iNOS (lung) activities were also investigated. To achieve this, an assay based on the ability of a whole brain or lung homogenates to convert [^{3}H]L-arginine to [^{3}H]L-citrulline was used (Faria et al., 1996). The iNOS activity in lungs was evaluated by pretreating the animals with *Escherichia coli* lipopolysaccharide (10 mg/kg, 12 h before). Data are reported as the mean ± SEM for four to five animals. The results were compared using analysis of variance (ANOVA) followed by the Bonferroni test. Values of $P < 0.05$ were considered as significant.

Intraperitoneal administration of SEB in the mice caused a significant peritoneal neutrophil accumulation (125 μg/cavity: 3.2 ± 0.5, 8.5 ± 1.8 and 3.5 ± 1.2 × 10^5 neutrophils/cavity at 4, 12 and 24 h after injection, respectively) as compared to PBS (1.6 ± 0.5, 1.1 ± 0.3 and 1.4 ± 0.3 neutrophils/cavity at 4, 12 and 24 h after injection, respectively; $n = 5$). For further studies, SEB was routinely administered at the dose of 125 μg/cavity and neutrophil influx evaluated 12 h later. Intravenous administration of L-NAME (100 μmol/kg), given immediately before intraperitoneal injection of SEB, reduced by 63% ($P < 0.05$) the neutrophil influx whereas D-NAME (100 μmol/kg) had no significant effect (Fig. 1). When given intraperitoneally, L-NAME (but not D-NAME) also significantly ($P < 0.05$) reduced SEB-induced neutrophil migration (Table 1). In addition, treatment of the animals with either the iNOS inhibitor aminoguanidine (400 μmol/kg, i.v., given immediately before SEB) or dexamethasone (0.5 mg/kg, s.c.; 1 h before SEB) significantly inhibited SEB-induced neutrophil influx (Fig. 1). In order to increase the peritoneal macrophage population, the animals were treated with thioglycollate (3% w/v, i.p.) and used 4 days later. By this time, the number of peritoneal macrophages increased from 4.4 ± 0.6 × 10^5 (non-stimulated cavity) to 36 ± 3.1 × 10^5 cells/cavity (stimulated cavity; $P < 0.05$). In these animals, intraperitoneal injection of SEB (125 μg) promoted a two-fold increase ($P < 0.05$) in the peritoneal neutrophil accumulation as compared to non-stimulated cavities (Fig. 1). In addition, L-NAME (100 μmol/kg; given intravenously immediately before injection of SEB) caused a significant reduction of the SEB-induced neutrophil influx, but this reduction (31 ± 6.5%) was markedly smaller as compared to the non-stimulated cavities (62 ± 8.0%, $P < 0.05$; see Fig. 1). Intravenous administration of D-NAME (100 μmol/kg; given immediately before injection of SEB) in thioglycollate-treated mice had no significant on SEB-induced neutrophil influx (Fig. 1). Similarly to L-NAME, treatment of the animals with either aminoguanidine (400 μmol/kg, i.v.) or dexamethasone (0.5 mg/kg, s.c.) significantly reduced the SEB-induced neutrophil accumulation in thioglycollate-treated mice (41 ± 7.9 and 38 ± 7.4%

Table 1
The effect of intraperitoneal administration of L-NAME or D-NAME on the SEB-induced neutrophil infiltration [L-NAME or D-NAME were administered intraperitoneally together with SEB (125 μg/cavity). Neutrophil migration was measured 12 h after the injection of toxin. The data represent mean ± SEM of five mice. $^{*}P < 0.05$ compared to the control groups]

Treatment	Neutrophils/cavity (× 10^5)
Control	8.5 ± 1.8
L-NAME 0.5 μmol/cavity	3.2 ± 0.1*
L-NAME 1.0 μmol/cavity	3.2 ± 0.4*
D-NAME 1.0 μmol/cavity	7.8 ± 0.6

reduction, respectively); however, these reductions were smaller ($P < 0.05$) than those of non-stimulated cavities (58 ± 4.2 and 67 ± 3.0%, respectively). In order to evaluate whether the in vivo doses of L-NAME (100 μmol/kg, i.v.) or aminoguanidine (400 μmol/kg, i.v.) were efficient to inhibit the cNOS and iNOS activities, the animals were treated with either compounds, and 12 h later the NOS activities in both brain (cNOS) and lung (iNOS) were determined. Intravenous administration of L-NAME significantly reduced the cNOS (2.8 ± 0.2 and 0.4 ± 0.1 pmol L-citrulline/min/mg protein for control and treated, respectively; $P < 0.05$) and iNOS activity (1.8 ± 0.2 and 1.1 ± 0.1 pmol L-citrulline/min/mg protein for control and treated, respectively; $P < 0.05$). Intravenous administration of aminoguanidine significantly reduced the iNOS activity (0.8 ± 0.2 L-citrulline/min/mg protein; $P < 0.05$), without significantly affecting the cNOS activity (2.3 ± 0.1 pmol L-citrulline/min/mg protein).

The role of NO on the leukocyte recruitment has been largely investigated, but results are still controversial. In mouse and human, inhibitors of NOS attenuate the neutrophil chemotaxis (Kaplan et al., 1989; Belenky et al., 1993; Menezes-de-Lima et al., 1997; Vianna and Calixto, 1998) whereas in rat they have no significant effect (Ferreira et al. 1996; 1998). Our results clearly show that the non-selective NOS inhibitor L-NAME (Moore et al., 1990) markedly reduced neutrophil migration induced by SEB into the mouse peritoneal cavity strongly indicating that NO modulates this phenomenon. At the dose used, L-NAME efficiently inhibited the activities of both the cNOS and iNOS isoforms, as expected. The failure of the inactive enantiomer D-NAME to affect the SEB-induced inflammatory responses confirms that inhibition by L-NAME was, in fact, due to NO synthesis inhibition. Additionally, the selective iNOS inhibitor aminoguanidine (Misko et al., 1993; Griffiths et al., 1993) significantly reduced SEB-induced neutrophil influx, supporting the concept that an iNOS isoform and, hence, a NO overproduction mediates this effect. Consistent with this, high serum levels of nitrite and nitrate were detected in mice after intraperitoneal injection of SEB (Florquin et al., 1994), and detectable expression of mRNA for iNOS was found in mouse endothelial cells treated with SEB (LeClaire et al., 1995). The findings that aminoguanidine markedly reduced the iNOS activity without affecting the cNOS activity indicate that inhibition of neutrophil influx is not secondary to a reduction of blood flow at the peritoneal microcirculatory bed. The SEB-induced neutrophil influx into the mouse peritoneal cavity was also reduced by dexamethasone. This is expected since glucocorticoid has been shown to inhibit the release of neutrophil chemotactic mediators such as arachidonic acid metabolites and cytokines (Fantuzzi and Chezzi, 1993; Klein et al., 1995), as well as the induction of iNOS (Di Rosa et al., 1990). The neutrophil influx induced by SEB was partly dependent on the presence of resident macrophages at the peritoneal cavity, confirming a previous study (DeSouza and Ribeiro-DaSilva, 1996). In peritoneal cavities with higher number of macrophages, treatment of mice with L-NAME, aminoguanidine or dexamethasone was less efficient to inhibit the neutrophil influx. Although this needs to be further clarified, we could speculate that in thioglycollate-stimulated cavities where the amount of inflammatory mediators (including NO) is largely increased, higher doses of NOS inhibitors and dexamethasone would be necessary. Interestingly, L-NAME (or aminoguanidine) and dexamethasone caused the same magnitude of inhibition in both non-stimulated and thioglycollate-stimulated cavity, indicating they are acting using the same pathway (NOS inhibition). This suggests, besides NO, other mediadors are implicated in the remaining neutrophil migration in animals treated with the above compounds. In conclusion, our results indicate that NO modulates SEB-induced neutrophil influx through the activity of an iNOS.

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Inducible Nitric Oxide Synthase in Rat Polymorphonuclear Leukocytes: role of insulin

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Running Title: Leukocyte NO Synthase Activity in Diabetes Mellitus

Classification: Hormones and Growth Factors

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§ *Abbreviations*: NO, nitric oxide; iNOS, inducible nitric oxide synthase; Tris, trizma base; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; EDTA, ethylenediaminetetraacetic acid; NADPH, nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; H_4B, (6R)-tetrahydro-L-biopterin; DTT, dithiothreitol; L-NAME, N^G-nitro-L-arginine methylester; L-NMMA, N^G-monomethyl-L-arginine; SDS, sodium dodecyl sulfate; NBT, 4-nitroblue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate.

ABSTRACT. Defective leukocyte-endothelial interactions are observed in experimental diabetes mellitus. Endogenous substances, including nitric oxide (NO), have anti-inflammatory effects within the vasculature by reducing leukocyte adherence to post-capillary venules. The purpose of this study was to examine the activity and expression of NO synthase in polymorphonuclear leukocytes from alloxan-induced diabetic rats. Glycogen-elicited peritoneal leukocytes were obtained from diabetic rats and matching controls 10, 30, and 180 days after alloxan (42 mg/kg, i.v.) or saline injection. NO synthase activity was determined by [^{3}H] L-citrulline assay method. Expression of the enzyme was investigated by Western blot analysis. Relative to controls, polymorphonuclear leukocytes obtained from diabetic rats presented: i) a 2-fold increase in the activity of NO synthase; ii) this was accompanied by an increase in the expression of the enzyme depicted by Western blot; iii) treatment of diabetic animals with NPH insulin (2 IU/day, for 3 days) reduced both the activity and expression of NO synthase to normal levels. Results presented suggest that overexpression of the inducible isoform of NO synthase by polymorphonuclear leukocytes might be responsible, at least in part, for the defects in leukocyte-endothelial interactions in diabetes mellitus.

KEY WORDS. polymorphonuclear leukocytes; nitric oxide synthase; diabetes mellitus

There is evidence to show that insulin is involved with the development of the inflammatory process [**1, 2**]. The early local exudative cellular reaction in an inflammatory lesion is impaired in alloxan induced rats due to a reduced migration of neutrophils to the inflamed area [**3**]. The finding is not dependent on the number of circulating leukocytes, hyperglycaemia alone, or hyperosmolality secondary to hyperglycaemia. A complete recovery is attained by treatment of the animals with insulin [3]. The number of leukocytes rolling along the venular endothelium of the microcirculation network is markedly reduced in diabetic rats. If a noxious stimulus is applied to induce a local lesion, leukocytes adhere to the vessel wall and accumulate in the connective tissue of normal animals in a pattern characteristic of the inflammatory reaction, whereas in diabetic rats the number of adhered leukocytes was reduced and only a few cells are found in the perivascular tissue. Reversal of the defective leukocyte-endothelial interactions is observed after treatment of diabetic animals with insulin [4, 5].

Several lines of evidence indicate that NO is an endogenous modulator of leukocyte adhesion in postcapillary venules [6], due to its ability to inhibit the expression of cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 [7]. NO is synthesized by a variety of cells from L-arginine, by a family of NO synthase enzymes. The endothelial [8] and neuronal enzymes [9] are constitutive isoforms dependent on calcium and calmodulin. The inducible isoforms, calcium-independent, are present in macrophages and others cells [10]. NADPH and cofactors, such as (6R)-tetrahydro-L-biopterin (H_4B), are required for enzyme activation [11, 12].

The aim of the present study was to evaluate the activity and expression of NO synthase in polymorphonuclear leukocytes from alloxan-induced diabetic rats.

MATERIALS AND METHODS

Materials

Alloxan monohydrate, oyster glycogen, Tris, HEPES, EDTA, NADPH, FMN, FAD, H_4B , DTT, L-NAME, L-NMMA, Dowex AG 50Wx8-400 resin, SDS, glycerol, bromophenol blue, and β-mercaptoethanol were purchased from Sigma Chemical Co. St. Louis, MO, USA. [^{3}H] L-arginine was from NEN Products, Boston, MA, USA. Liquid scintillation Ultima Gold was from Packard, USA. NPH insulin (Iolin®) was from Biobrás, São Paulo, Brazil. NBT and BCIP were obtained from Bio-Rad, USA. Nitro-cellulose membrane was from Amersham, USA. Anti-NO synthase antibody (mouse IgG_1 anti-rat iNOS), alkaline-phosphatase conjugated rabbit anti-mouse IgG, and the positive control (mouse macrophage iNOS) were from Transduction Laboratories, KY, USA.

Animals

Male Wistar rats weighing 180 to 200 g at the beginning of the experiments were used. The animals were allowed a standard pellet diet and free access to water and maintained at 23° C under a cycle of 12 h light/dark. All experiments were in accord with ethical principles in animal research adopted by the Biomedical College Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/University of São Paulo- Ethical Committee for Animal Research (CEEA).

Induction and treatment of Diabetes Mellitus

Diabetes mellitus was induced by the intravenous injection of 42 mg/kg alloxan dissolved in physiological saline. Control rats were injected with physiological saline alone. Ten, 30, or 180 days thereafter, the presence of diabetes was verified by blood glucose concentrations > 200 mg/dL, determined with the aid of a blood glucose monitor (Advantage®, Eli Lilly, São Paulo, Brazil) in samples obtained from the cut tip of the tail. A group of diabetic animals were treated with NPH insulin 2 IU/day, s.c., for 3 days before the experiments.

Isolation of peritoneal neutrophils

Neutrophils were obtained from the peritoneal cavity of the animals 4 h after the injection of 20 mL of sterile 1% oyster glycogen in physiological saline [13]. Erythrocytes were removed by hypotonic lysis. Final cell suspension contained 95- 98 % neutrophils. Cell viability was assessed by Eosin Y exclusion method.

NO Synthase activity

NO synthase activity was measured by the [^{3}H] L-citrulline assay method as described previously [14] with slight modifications. Neutrophils (100 x 10^6 cells/mL) resuspended in HEPES buffer (20 mM HEPES, 2 mM $CaCl_2$, 0.32 M sucrose, 1mM DTT , pH 7.4) were lysed with the aid of a sonifier (Thorton, São Paulo, Brazil), and centrifuged at 500 g for 10 min. Aliquots (100 µL) from the supernatant were incubated with 200 µL HEPES buffer (50 mM HEPES, 1 mM EDTA, 1.25 mM $CaCl_2$, pH 7.4), containing 4 µM FAD, 4 µM FMN, 4 µM H_4B, 11 mM NADPH and 1 µCi [^{3}H] L-arginine for 60 min at 37°C . The reaction was stopped by the addition of 20 µl cold buffer containing 20 mM L-arginine and 50 mM EDTA. The reaction mixture was applied to Dowex AG 50Wx8-400 (Na^+ form) resin columns and eluted with HEPES buffer (20 mM, pH 5.5) and water (v/v). The radioactivity corresponding to [^{3}H] L-citrulline content in the sample was measured with liquid scintillation counting. Protein concentration was determined by the Bio-Rad Protein Assay [15]. Results are presented as pmoles citrulline/ mg protein/ min.

Tests were performed in the absence of NADPH and calmodulin and in the presence of NO synthase inhibitors, such as L-NAME and L-NMMA, at the concentrations of 10, 100, and 1000 µM.

Western blot

Samples obtained from lysed neutrophils, as described above, were diluted (1 mg protein/mL) in Tris buffer (62.5 M Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue) and heated at 100°C for 5 min. Proteins (10-20 μg) were separated on 7% SDS-PAGE [16] and transferred to nitrocellulose membranes during 2 h (150 mA) in Tris-glycine buffer (25 mM Tris, 192 mM glycine) containing 0.1% SDS and 20% methanol. Membranes were incubated with Tris buffer saline (TBS, 200 mM Tris, 1.37 M NaCl, pH 7.6) containing 5% skimmed milk, followed by overnight incubation at 4°C with the primary monoclonal antibody (mouse IgG_1 anti-rat iNOS, 1:2500). After extensive washings in TBS, the nitrocellulose membranes were incubated with the secondary antibody (rabbit anti-mouse IgG conjugated to alkaline phosphatase, 1:1500) for 2 h at room temperature. The immunoreactive proteins were visualized with NBT/BCIP. The densitometric quantitation of iNOS expression was performed using the Sigma Gel Software-Jandel Scientific (Sigma Chemical Co. St. Louis, MO, USA). Results were presented as arbitrary units.

Data analysis

Results are presented as means ± SEM. When appropriate, the data were statistically analysed by Student's t test or analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. $p < 0.05$ was considered significant.

RESULTS

NO synthase activity in alloxan induced diabetic rats

Relative to controls, the activity of NO synthase in polymorphonuclear leukocytes was markedly increased in rats rendered diabetic by the injection of alloxan. Estimates made 10, 30 or 180 days after alloxan administration showed that the increase in NO synthase activity was comparable in all groups of animals tested. The finding was not dependent on the number of neutrophils recovered from the peritoneal cavity of the animals. Values were (mean ± SEM): $1.30 \pm 0.07 \times 10^8$ cells and $1.40 \pm 0.11 \times 10^8$ cells in control and diabetic rats, respectively. Blood glucose concentrations were sharply elevated and a significant reduction in body weight gain was observed in diabetic animals during these intervals. Results are summarized in Table 1.

Tests performed in the absence of some cofactors showed that the activity of the enzyme did not change in the absence of calmodulin, but calcium absence reduced NO synthase activity by 30-35 % in polymorphonuclear leukocytes from both diabetic and control animals. In the absence of NADPH, enzyme activity was almost completely abolished. Whatever the situation NO synthase activity in polymorphonuclear leukocytes from diabetic rats was twice as that observed in controls. Results are illustrated in Figure 1. Similar results were observed upon inhibition of NO synthase by L-NAME and L-NMMA. Both substances caused a dose-dependent inhibition of NO synthase, the remaining enzyme activity levels being higher in diabetic rats than in controls (Fig. 2).

Role of insulin on NO synthase activity

In order to verify the relationship between NO synthase activity and the circulating level of insulin, animals rendered diabetic by the injection of alloxan 10 days before were given 2 IU NPH insulin each evening for the last 3 days of the period. A complete normalization of the NO synthase activity was observed in diabetic rats treated with insulin. Values attained matched those observed in controls. Results are illustrated in Figure 3. Blood glucose levels decreased from (mean ± SEM) 447 ± 15 before treatment to 285 ± 28 mg/dL after insulin treatment ($p < 0.0001$).

Western blot analysis performed with the lysates from polymorphonuclear leukocytes showed a strong immunoreactivity for the inducible NO synthase in the diabetic group, the level of protein expression decreasing after treatment of diabetic animals with insulin (Fig. 4).

DISCUSSION

Results presented suggest that the activity of NO synthase in polymorphonuclear leukocytes is regulated by insulin. The suggestion is supported by the following observations. First, the activity of NO synthase was markedly increased in leukocytes obtained from alloxan-induced diabetic rats. Second, this was accompanied by an increase in the level of enzyme expression. Third, both the activity and expression of NO synthase returned to normal values after treatment of diabetic rats with insulin.

Determinations of NO synthase activity by the [^{3}H] L-citrulline assay method showed that enzyme activity in peritoneal leukocytes from diabetic rats was approximately twice the values observed in controls. Differences between diabetic and control groups ranged from 1.5 to 2.5 times. This was observed from the early stages of diabetes, remaining up to 180 days after alloxan injection. Similar results were obtained from the analysis of NO synthase expression by Western blot. The lysates prepared from polymorphonuclear leukocytes of diabetic rats showed a strong immunoreactivity for the inducible isoform of NO synthase (130 kDa). Tests performed to characterize NO synthase in peritoneal leukocytes showed that the enzyme activity is dependent on calcium but not on calmodulin. NO synthase purified from rat polymorphonuclear leukocytes is known to be dependent on calcium, NADPH and H_4B [17, 18]. A 130 kDa protein and a 22 kDa linked protein were purified from human neutrophils by SDS-polyacrilamide gel electrophoresis [19] and was characterized in these cells upon stimulation with interleukin-1, tumor necrosis factor-α, and interferon-γ [20] as well in rat neutrophils in a model of endotoxic shock [21]. Furthermore, we clearly

demonstrated a dose dependent effect with both inhibitors of NO synthase, L-NAME and L-NMMA, on the activity of NO synthase in rat polymorphonuclear leukocytes.

In both series of experiments, testing enzyme activity in the absence of some cofactors or in the presence of L-NAME and L-NMMA, the activity of NO synthase in leukocytes from diabetic rats was approximately twice the activity of the enzyme observed in matching controls. Values returned to normal levels after treatment of diabetic animals with insulin. Daily injections of NPH insulin had to be administered for at least 3 days before experiments. Insulin treatment clearly was not sufficient to maintain normal blood glucose levels in these animals. Accordingly, the increase in NO synthase activity might be primarily linked to continuing insulin deficiency rather than to secondary hyperglycaemia occurring in diabetic animals. Adequate concentrations of insulin seem to be required for the normal function of endothelial cells and leukocytes during the course of the inflammatory process. The local exudative cellular reaction in a inflammatory lesion, including the carrageenan-induced pleurisy [3], the allergic pleurisy [22], and the allergic airway inflammation [23] depends on the availability of insulin.

Recent studies support the idea that insulin might regulate the activity of NO synthase. Macrophage NO production (NO_2 levels) and NO synthase mRNA expression are elevated in rats and mice made diabetic by streptozotocin injection [24]. Increased expression of endothelial cell NO synthase, on diabetic rat kidney, was prevented by treatment of the animals with L-NAME or insulin [25]. We presently demonstrated that the activity and expression of the inducible isoform of NO synthase in polymorphonuclear leukocytes are increased in alloxan-induced diabetic rats. The ability of insulin to normalize enzyme activity

and expression is an indication that the alteration observed is a consequence of the diabetic state.

Several lines of evidence implicate NO as an endogenous inhibitor of leukocyte adhesion in venules. NO synthase inhibition elicit recruitment of adherent leukocytes [26, 27]; NO donors (nitroprusside) attenuate or prevent leukocyte adherence induced by different inflammatory stimuli [28]; superoxide, by reacting with NO, promotes leukocyte adherence [29]. Previous studies showed that defective leukocyte-endothelial interactions and leukocyte chemotaxis are observed in experimental diabetes mellitus [3-5, 30]. Any dysfunction in the sequence of events leading to the ability of leukocytes to migrate efficiently in response to inflammatory stimuli could result in an increased susceptibility of the host to infectious diseases [31, 32]. It is well established that certain infections occur almost exclusively in diabetic patients and many diabetic patients have a worse prognosis once infection is established [1-2, 33].

Results presented suggest that overexpression of the inducible isoform of NO synthase by polymorphonuclear leukocytes might be responsible, at least in part, for the defects in leukocyte-endothelial interactions in diabetes mellitus, which represents an aggravating factor for host defence in the first stages of infection.

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TABLE 1. General characteristics of the animals and NO synthase activity in polymorphonuclear leukocytes

Animals	Body weight gain (g)	Blood glucose (mg/dL)	NO synthase activity (pmol/mg/min)	n
Diabetic (10 days)	5 ± 3 †	444 ± 12 †	343.86 ± 16.29 *	7(12)
Matching controls	55 ± 3	105 ± 4	171.98 ± 19.88	7(11)
Diabetic (30 days)	23 ± 13 †	367 ± 24 †	191.17 ± 4.26 †	3(6)
Matching controls	120 ± 4	97 ± 5	133.30 ± 1.02	3(6)
Diabetic (180 days)	104 ± 14 ‡	371 ± 11 †	268.10	1(3)
Matching controls	232 ± 11	108 ± 3	105.80	1(3)

Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10, 30 or 180 days before. Values are means ± SEM for n assays. Figures within parentheses indicate the number of animals used in each group. * $p < 0.0001$; † $p < 0.001$; ‡ $p<0.01$ vs matching controls.

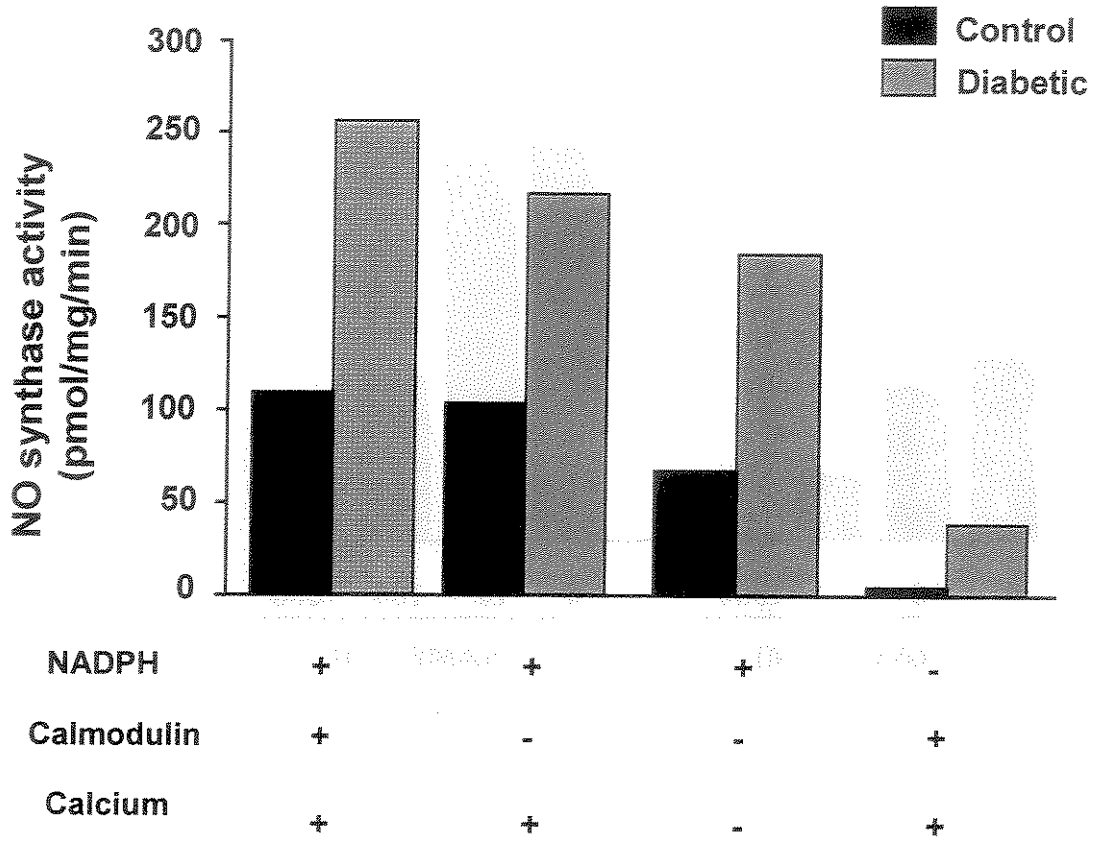
Legends for figures

FIG. 1. Effect of calmodulin (400 U/ml), Ca^{2+} (2 mM) and NADPH (11 mM) on the activity of NO synthase ([^{3}H] L-citrulline assay method) in polymorphonuclear leukocytes from diabetic rats and matching controls. Animals were rendered diabetic by injection of alloxan (42 mg/kg, i.v.) 10 days before. Glycogen-elicited peritoneal leukocytes from 3 to 4 rats were pooled, each animal yielding approximately 100 x 10^6 cells. Values presented are means of 2 independent experiments.

FIG. 2. Inhibition by L-NAME and L-NMMA of NO synthase activity ([^{3}H] L- citrulline assay method) in polymorphonuclear leukocytes from diabetic rats and matching controls. Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before. Glycogen-elicited peritoneal leukocytes from 3 to 4 rats were pooled, each animal yielding approximately 100 x 10^6 cells. Values presented are means of 2 independent experiments.

FIG. 3. NO synthase activity ([^{3}H] L- citrulline assay method) in polymorphonuclear leukocytes from diabetic (n=6), diabetic rats treated with insulin (n=8) and matching controls (n=7). Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before. Insulin (NPH, 2 IU/day, s.c.) was given for the last 3 days before testing. Glycogen-elicited peritoneal leukocytes from 2 to 3 rats were pooled, each animal yielding approximately 100 x 10^6 cells. Values are means ± SEM of 3 to 4 experiments in each group. * $p < 0.05$ vs other values.

FIG. 4. Western blot analysis was performed with the lysates from polymorphonuclear leukocytes. Cells were obtained from diabetic rats (D), diabetic rats treated with insulin (DI) and matching controls (C). Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before. Insulin (NPH, 2 IU/day, s.c.) was given for the last 3 days before testing. Glycogen-elicited peritoneal leukocytes from 2 to 3 rats were pooled, each animal yielding approximately 100 x 10^6 cells. The inducible NO synthase (iNOS) from mouse macrophages was used as positive control. Densitometric analysis (inset) was performed using the Jandel Scientific Software (Sigma Gel - USA).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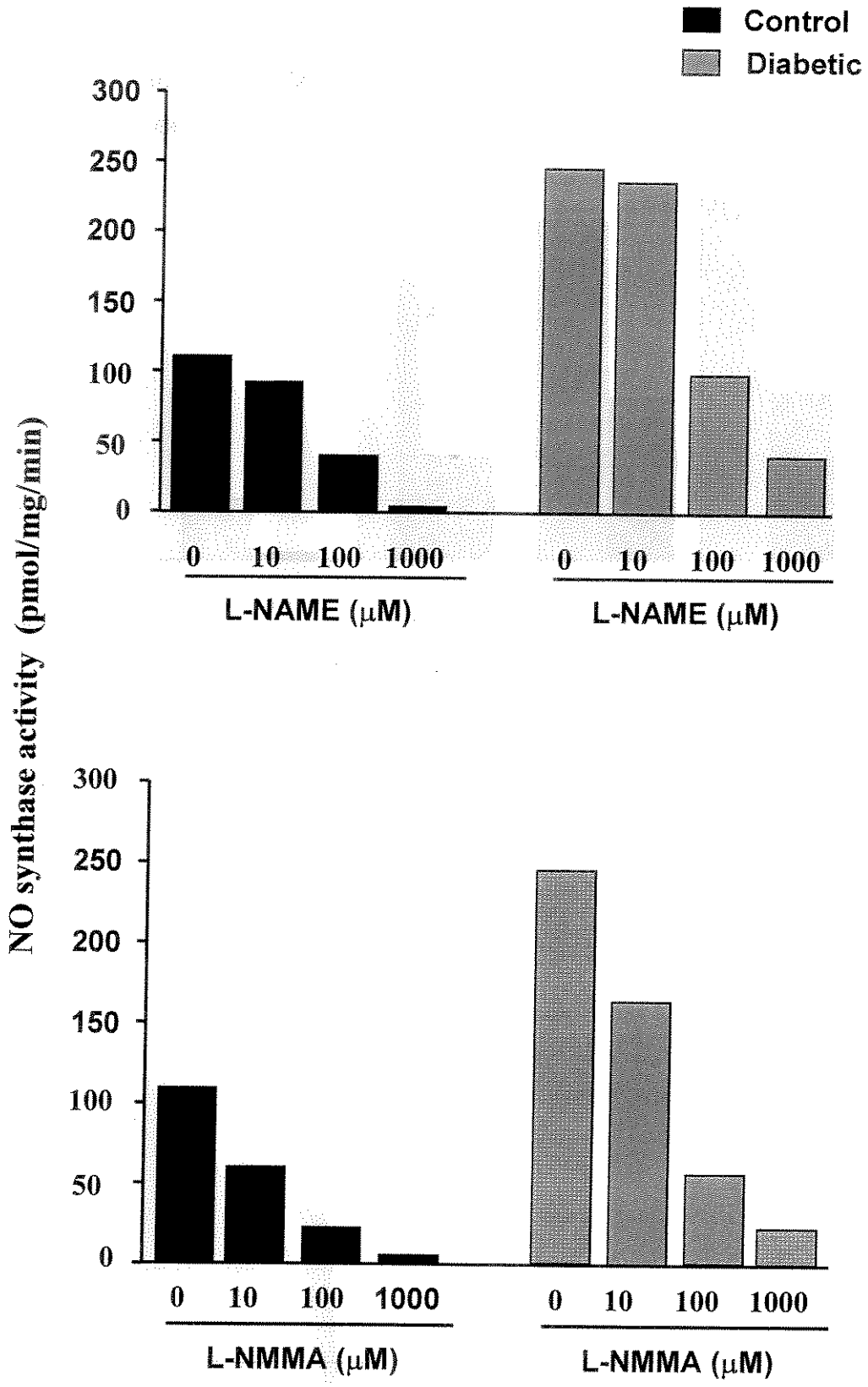
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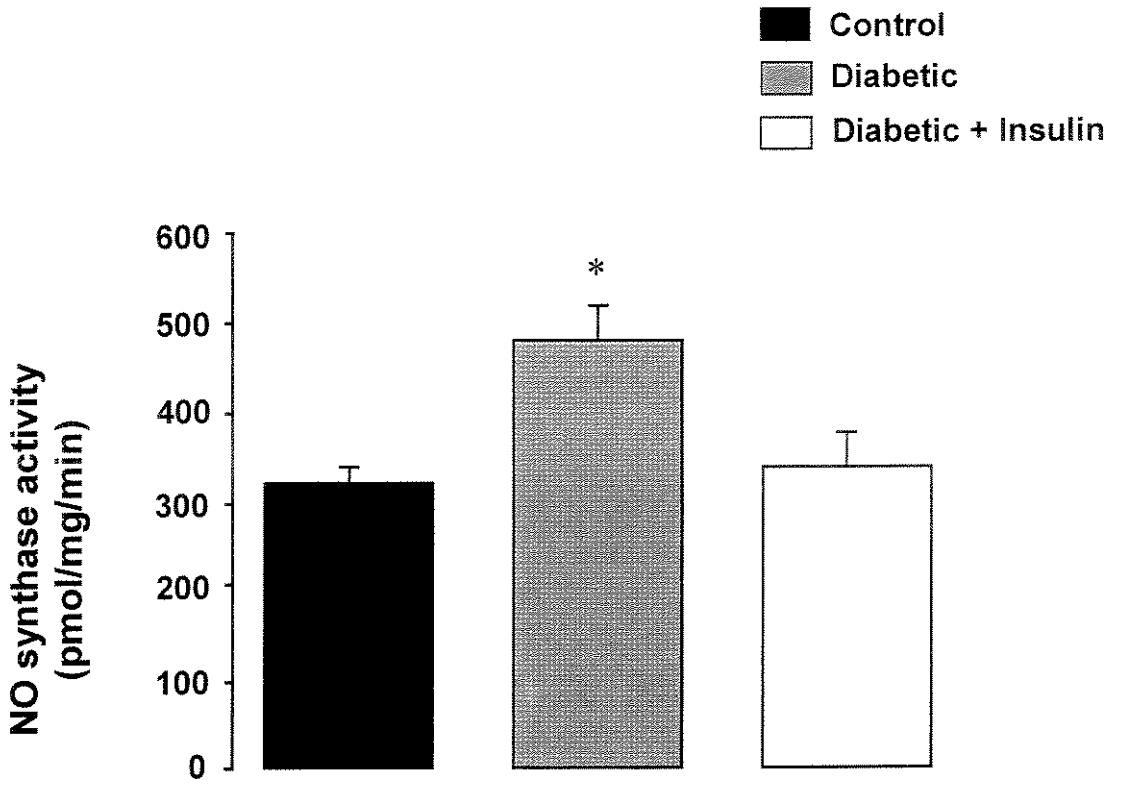
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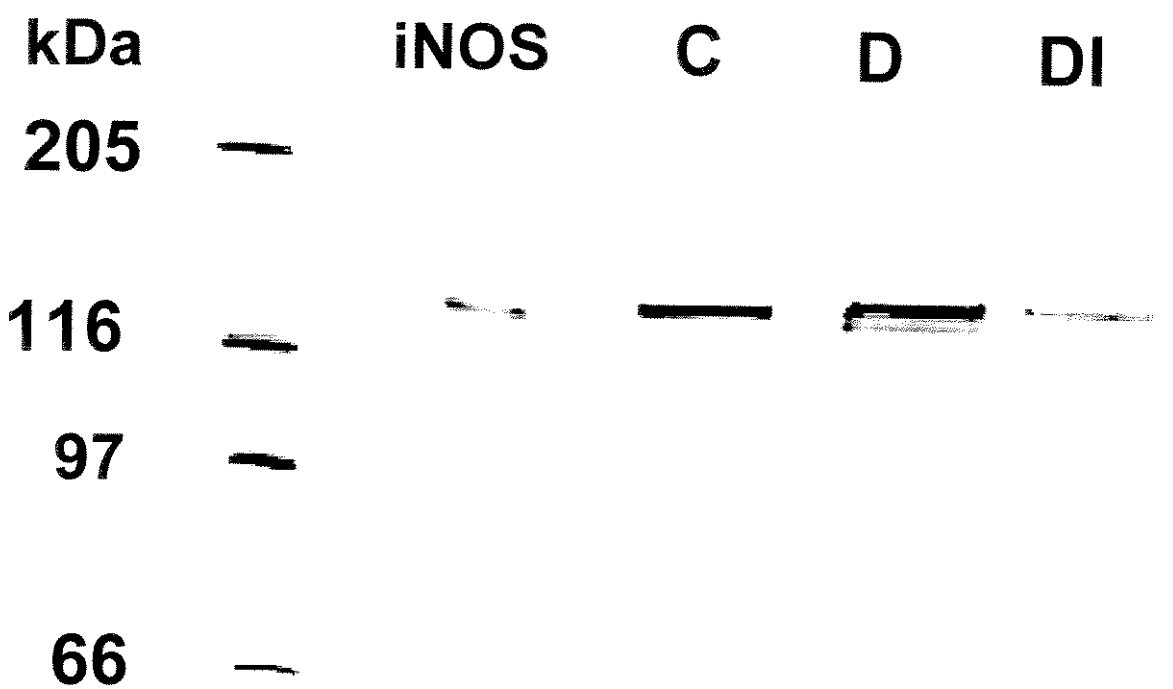
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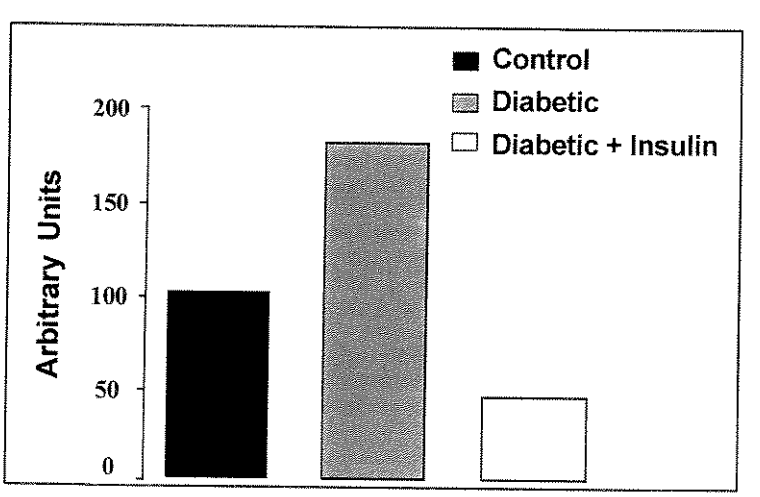
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Diabetic
Diabetic + Insulin
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NEUROSCIENCE LETTERS

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Title: Expression of neuronal isoform of nitric oxide synthase in spinal neurons of neonatal rats after sciatic nerve transection.

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USA.

Dear Professor Ralston,

Thank very much for informing us of the acceptance of our manuscript. We did the suggested corrections numbered 1-5 and rewrote the paragraph of P.8 starting with '**Another possibility...**' to include the reference by Wu et al. (1995). We also found that the reference by Sharma et al. (1998) could be useful. This takes the total of cited papers to 22. I hope this will still be acceptable to the journal regulations.

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EXPRESSION OF NEURONAL ISOFORM OF NITRIC OXIDE SYNTHASE IN SPINAL NEURONS OF NEONATAL RATS AFTER SCIATIC NERVE TRANSECTION

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ABSTRACT

Motoneuron death induced by sciatic nerve transection in neonatal rats has been related to induction of the neuronal isoform of nitric oxide synthase (nNOS), a diaphorase of which one of the cofactors is NADPH. We transected the sciatic nerve of neonatal rats (P2) and examined nNOS expression by immunostaining in neurons of the sciatic pool and of other spinal levels on the fifth day after surgery. No correspondence was observed between the surviving motoneurons and nNOS positive cells. The appearance and distribution of nNOS positive neurons at all spinal levels and laminae were similar to those of adult animals. These results are at variance with previous studies which showed correlation between motoneuron loss after axotomy and number of NADPH-diaphorase positive motoneurons after sciatic transection.

Key words: Nitric oxide synthase; Neonatal rat; Motoneuron; Sciatic nerve; Axotomy; Neuronal death; Spinal neurons

Transection of peripheral nerves of rodents during the first postnatal week is known to cause death of numerous motoneurons [6, 11, 12, 15]. A possible mechanism might be intense free radical production such as nitric oxide (NO) and its derivatives [5, 16, 22]. NO is formed in the cytoplasm by the neuronal isoform of nitric oxide synthase (nNOS) and may interact with various intracellular molecules, including other free radicals like superoxide (O_2^-). This results in peroxinitrite ($ONOO^-$) which may cause lipid peroxidation of membranes, inhibition of mitochondrial respiration and apoptosis [13]. NOS is a NADPH-dependent diaphorase [8] and histochemically demonstrable NADPH-diaphorase activity in nervous tissue has been interpreted as evidence of nNOS expression [1, 4, 18, 19, 22].

Clowry (1993) [4] demonstrated that sciatic nerve transection in newborn Wistar rats (P1) increases the proportion of NADPH-diaphorase positive motoneurons in the dorsolateral region of the ventral horn of the lumbar enlargement between 4 and 18 days after the lesion with a peak on the 5th day. As some of these neurons showed nucleolar fragmentation it was proposed that the diaphorase detected during motoneuron death would be nNOS.

With the aim of further evaluating nNOS expression in this experimental model we marked the enzyme by immunohistochemistry on the fifth day after axotomy when neuronal death and the proportion of NADPH-diaphorase positive neurons were found to be maximal [4]. We also examined the morphology and distribution

of nNOS positive neurons in other spinal levels (i. e. cervical, thoracic and sacral).

Wistar rats (n=8) at an age of 2 days postnatally (P2) were anesthetized by hypothermia. The sciatic nerve was transected unilaterally at mid-thigh level. A short segment (approximately 3 mm) of the distal stump was removed. After surgery, the rats was returned to their mother and allowed to survive for 5 days.

Animals were anesthetized with sodium pentobarbital 3% (0,1 ml/20g; i.p.) and perfused through the heart with saline followed by 40 ml of freshly prepared 4% paraformaldehyde in phosphate buffered saline.

Spinal cords were dissected into segments corresponding to cervical (C5-C7), thoracic (T4-T10), lumbar (L3-L6) and sacral (S2-S4) levels and postfixed in the same fixative for 24 h at 4° C. The specimens were rinsed with water, dehydrated in ethanol and embedded in paraffin as serial blocks as described by Beesley and Daniel (1956) [2]. Serial sections (8μm) were cut from each block. In every four sections the first was stained with cresyl violet (Sigma), the second was reacted for nNOS immunohistochemistry and the third and fourth sections were not collected.

For immunostaining sections were incubated in primary antibody to nNOS (rabbit polyclonal antibody; Transduction Laboratories – KY, cat # N31030) overnight, at room temperature (20° C). This antibody was applied in 1:50 dilution in 0,1M PBS containing 16% normal goat serum and 0.3 % Triton X-100. Sections were then incubated in biotinylated secondary antiserum (anti-rabbit; Rockland – PA, cat # 611-1602) for 1 h at 20° C and, following that, were incubated in ABC

reagents (Vectastain ABC kit; Vector - CA) for 1 h. Finally, sections were incubated in diaminobenzidine (0,5 mg/ml) and H_2O_2 (10 µl) for 25 minutes. In control experiments, the primary antibody was omitted. Immunoreactivity to nNOS was examined in neurons and neuropil in all Rexed's laminae of the different spinal cord levels.

After cresyl violet staining large neurons of the ventrolateral group (IX laminae) containing a clearly-visible nucleolus were counted on every section at the lumbar level as described previously [7]. The unoperated contralateral side of the spinal cord was used as control. The percentage of motoneuronal loss compared with the control side, was calculated. Statistical analysis was performed using the Student t - test.

Immunostaining for nNOS in neurons was observed in the cell body and processes but not in the nucleus. Densest staining was seen in laminae I-IV, from cervical through sacral levels. The neuropil in laminae I and III reacted more intensely than in II, reproducing the bilaminar pattern described by others in the dorsal region of adult rat spinal cords [19]. Most nNOS positive neurons in the deeper dorsal horn were single cells in laminae IV and V, usually bipolar or polygonal (Fig. 1A). At thoracic and sacral levels, lamina VII contained groups of intensely reacting neurons linked by bundles of cell processes to neurons situated around the central canal (lamina X). Isolated projections towards the ventral horn were also seen. In adult rats, immunohistochemical studies identified the lamina

VII group of neurons as constituents of preganglionic sympathetic (thoracic) and parasympathetic (sacral) columns [1, 19]. Numerous nNOS positive neurons were situated around the central canal (lamina X) at all levels. These were mostly bipolar, arranged singly or in small groups amid a network of reactive varicose fibers and occasionally vessels (Fig. 1B).

At the lumbar ventral horns, some neurons were stained at both the control and axotomized sides. These were mostly bipolar, arranged singly or in small groups in laminae VII, VIII and IX. Large polygonal neurons were identified in the ventral aspect of laminae VII and IX. nNOS immunoreactivity for all these cells was small to absent. No marked cells had morphological features indicative of motoneurons (Fig. 1C and Fig. 1D).

Cresyl violet stained sections were used for counting lumbar motoneurons of the eight animals, which yielded a 60% reduction on the axotomized (61±9; mean±SEM), compared to the control side (151±15) ($p<0.001$). These sections were also helpful in the identification and morphological evaluation of immunostained neurons (Fig. 1E and Fig. 1F).

Our study in neonatal rats showed nNOS positive spinal neurons most notably around the central canal, in the lateral group of lamina VII (presumably preganglionic autonomic) and bipolar neurons in laminae IV, V, VII - IX. These results agree with those reported for adult animals [1, 19] and suggest that at P7 this enzyme is constitutively expressed with no deleterious function. Among

physiological functions attributed to NO in spinal neurons are the modulation of synaptic transmission and neuroplasticity, both of importance during CNS maturation [9]. Kalb and Agostini (1993) [10] observed NOS expression in cells of the lumbar cord of P7 rats, thought to be interneurons, and suggested that enzyme activity in them may be transitory and related to development of the adjacent motoneurons of the sciatic pool. NOS might contribute to the development of the molecular phenotype of the motoneurons which is acquired early in the neonatal period. However, maintenance of such phenotype would be independent of the enzyme as it does not change in adult animals subjected to NOS inhibitors.

We did not observe differences in immunostaining of lumbar neurons between operated and control sides five days after axotomy. There was no evidence that nNOS is more expressed in axotomized motoneurons, or that nNOS positive neurons are more numerous on the injured side, although counting of immunolabeled neurons was not attempted. There was no morphological correspondence between motoneurons identified in Nissl-stained material and nNOS positive neurons in adjacent sections. Our results therefore do not support previous observations that nNOS would be expressed by immature motoneurons 5 days after axotomy [4]. The observations of Piehl et al. (1998) [17] also do not provide evidence that damaged motoneurons express nNOS, as these authors did not find nNOS mRNA expression in newborn rat lumbar motoneurons (P3) at 1, 3 or 7 days after axotomy. Furthermore Kalb and Agostini (1993) [10] did not

observe coincidence between undamaged motoneurons retrogradely labeled by Fast Blue and NOS-positive lumbar neurons in P7 rats. The latter cells did not have shape or size of motoneurons and were distributed around Fast Blue labeled neurons which suggests they were probably premotor interneurons.

It is therefore possible that nNOS expression is not altered by axotomy and that the diaphorase demonstrated by Clowry (1993) [4] is different from nNOS [3]. Liuzzi et al. (1993) [14] studied NOS expression in the dorsal horn of neonatal rats (P7) using the histochemical tecnique for NADPH-diaphorase and found intense marking in lamina II and almost nil in laminae I and III. That is the opposite of what we found using immunohistochemistry for nNOS, which indicates that the enzymes demonstrated by each technique may be different as suggested by Callsen-Cencic et al. (1999) [3].

Another possibility is that nNOS expression did occur but this was fatal to the cells before P7 when the material was collected so the enzyme was no longer demonstrable. For instance, He et al. (1996) [7] observed NADPH-diaphorase activity in motoneurons of newborn mice after sciatic transection at P1 but not at P5. Wu et al. (1995) [21] also employed histochemical demonstration of NADPH-d to analyse NOS expression in spinal rat motoneurons after C7 ventral root avulsion in newborn and early postnatal rats and observed extensive diaphorase activity on the side of the lesion. The staining occurred earlier and was more intense in younger animals. The reason why these authors found NADPH-diaphorase activity

after axotomy and we did not is unclear. It is possible that the differences could be due to the types of lesion employed. Nerve root avulsion is severer than nerve transection because it occurs closer to the cell body and no root stump is left. The proximal stump of transected nerves contains Schwann cells that produce neurotrophic factors which may increase motoneuron survival, possibly by regulating nNOS expression or activity [20, 21].

Function of nNOS in normal and pathological spinal cord is still little understood, in part due to uncertainties in enzyme demonstration by available techniques. Using immunohistochemistry instead of histochemistry for diaphorase activity we found no evidence that alterations of nNOS expression underlie motoneuron demise following axotomy.

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Figure 1

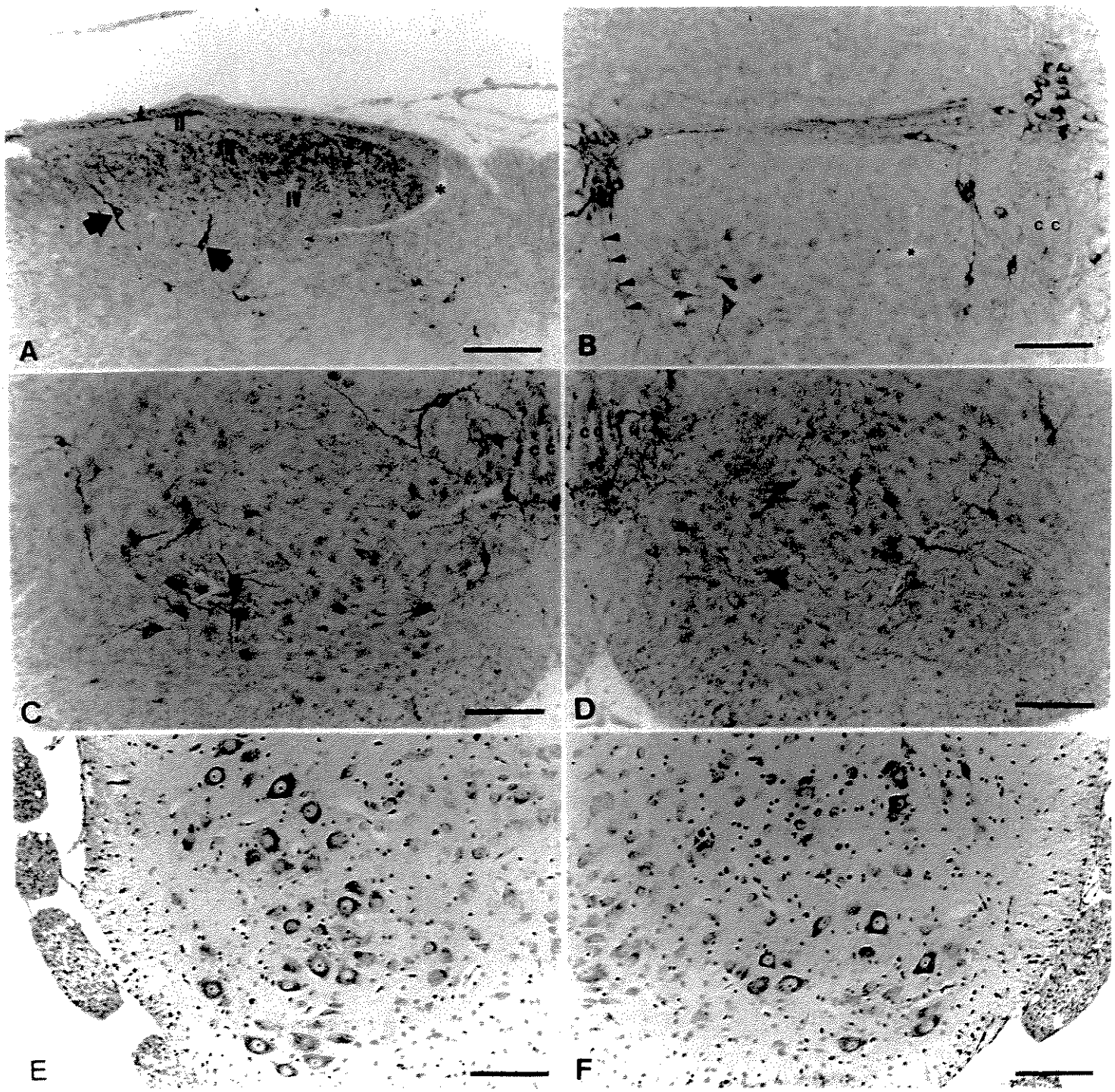
A. Bilaminar pattern of immunolabeling for nNOS in the dorsal thoracic spinal cord. Large bipolar neurons in lamina IV (arrows).

B. nNOS immunohistochemistry in transverse sections of thoracic cord. Neurons of lateral column and around the central canal (cc) are joined by nNOS positive fibers. Other isolated nNOS positive fibers are seen towards the ventral horn (arrows).

C, D. nNOS immunohistochemistry in ventral horn neurons of non-injured (C) and injured (D) sides. nNOS positive neurons of different shapes and sizes are seen in laminae VIII and IX, with predominance of bipolar neurons. The number of labelled neurons is larger in the non-injured side. No cells morphologically suggestive of motoneurons are labelled on either side.

E, F. Cresyl violet stained lumbar motoneurons of non-injured (E) and injured (F) sides. No chromatolysis is seen on either side.

Scale bar = 100μm; (I, II, III, IV: Rexed laminae; CC: central canal; * blood vessel.).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Subject: En: paper for Toxicon
Date: Mon, 9 Apr 2001 21:55:46 -0300
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From: Toxicon (A.L. Harvey) <toxicon@strath.ac.uk>
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Sent: Tuesday, April 03, 2001 10:14 AM
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> *Dear Dr de FP Teixeira*
>
> *I am pleased to inform you that your paper has been accepted for Toxicon.*
> *It has been sent to the publishers and you will receive proofs in due course.*
>
> *Alan Harvey*
>

Bothrops asper AND *Bothrops jararaca* SNAKE VENOMS TRIGGER MICROBICIDAL FUNCTIONS OF PERITONEAL LEUKOCYTES *in vivo*

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Short title: *Bothrops* snake venom triggers leukocyte functions.

Abstract: Venoms from snakes of the genus *Bothrops* cause pronounced local effects in the victims. These alterations result not only from the direct toxic action of venom components, but also from the prominent inflammatory reaction associated with these envenomations. In this study we investigated the ability of *Bothrops asper* (BaV) and *Bothrops jararaca* (BjV) venoms to induce cellular influx and microbicidal functions in leukocytes. BaV and BjV (5 μg/animal) caused a long lasting infiltration of leukocytes (3 to 48 h) when injected into mouse peritoneal cavity. Both venoms increased phagocytosis and production of hydrogen peroxide (H_2O_2) by polymorphonuclear (PMN) and mononuclear (MN) peritoneal leukocytes. In addition, nitric oxide (NO) production by macrophages was also enhanced after the venom injections. This effect was inhibited by treating animals with L-NAME and aminoguanidine, thus suggesting the induction of iNOS synthesis by the venoms. Western blot analysis confirmed the expression of iNOS in macrophages. BaV and BjV injection led to increased levels of IFN-γ at the site of inflammation. Since IFN-γ is an effective inducer of iNOS expression, an indirect action of the venoms on iNOS expression can be proposed. A marked formation of nitrotyrosine-containing proteins was also observed in macrophage homogenates. Based on these results, we suggest that reactive oxygen and nitrogen-derived species are involved in the pathogenesis of the local tissue damage characteristic of *Bothrops* sp envenomations.

Keywords: Snake venoms; Leukocyte functions; Nitric oxide; Hydrogen peroxide; Peroxynitrite

1. Introduction

In Latin America, the majority of snakebites are caused by species of the genus *Bothrops* (Cardoso, 1985; Gutiérrez, 1995). *B. jararaca* and *B. asper* are responsible for most snake poisonings in Brazil and Costa Rica, respectively. Envenomations caused by these species result in severe local tissue damage, with haemorrhage, pain, myonecrosis and inflammation (Rosenfeld, 1971; Gutierrez et al., 1984, 1986; Trebien and Calixto, 1989; Flores et al., 1993). It has been shown that *Bothrops* venoms induce a significant leukocyte accumulation at the site of tissue damage (Flores et al., 1993; Acosta De Pérez et al., 1996; Farsky et al., 1997). However, little is known about the activation state of these cells at the site of *Bothrops* venom injection. Leukocytes play essential roles in the host defense against offending agents and are key mediators in the inflammatory response (Miller and Britigan, 1995; Ley, 1996). On exposure to a pathogen, leukocytes respond with phagocytosis followed by generation and release of several microbicidal products (Rohn et al., 1999).

One of the most important metabolic pathways activated by phagocytosis in leukocytes is the respiratory burst, characterized by an increased oxygen consumption and consequent generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). In this metabolic pathway, oxygen is enzymically reduced through a series of one-electron reductive reactions catalyzed by a NAD(P)H oxidase system (Pick and Mizel, 1981; Russo et al., 1989; Caron and Hall, 1998). Another pathway stimulated during phagocytosis involves the generation of nitric oxide (NO) and related reactive nitrogen intermediates (RNI) by macrophages and neutrophils. NO is enzymically synthesized from L-arginine by a family of enzymes generically known as nitric oxide synthases (NOS; Nathan, 1992; Chan, et al., 1995). Type II or inducible nitric oxide synthases (iNOS) expression occurs in

several pathological processes and its synthesis is induced by some cytokines following immune activation (Moncada, 1992; Hogaboam et al., 1997).

Despite the well demonstrated role of leukocytes and their reactive products in the pathogenesis of a variety of diseases, little is known on their activation in snake venom-induced tissue damage. In the present work we investigated the effect of *B. asper* and *B. jararaca* venoms on leukocyte recruitment and microbicidal functions at the site of injection, as well as the mechanisms involved in such actions.

2. Materials and Methods

2.1. Venom

Lyophilized crude venoms of *B. asper* (BaV) and *B. jararaca* (BjV) were supplied by Clodomiro Picado Institute, San José, Costa Rica and Herpetology Laboratory from Butantan Institute, São Paulo, Brazil, respectively. The venoms were solubilized in 0.9% NaCl solution and subsequently filtered through sterilizing membranes (0.22 μm pore size; Millipore Ind. Com. Ltda., Brazil) before use.

2.2. Animals

These studies were approved by the Experimental Animals Committee of Butantan Institute in accordance with the procedures laid down by the Universities Federation for Animal Welfare. Male Swiss mice (19-20 g) were housed in temperature-controlled rooms and received water and food *ad libitum* until used.

2.3. Induction of inflammatory reaction

BaV and BjV (5 μg/mouse) dissolved in 1 ml of sterile saline were injected intraperitoneally (i.p.). Control animals received 1 ml of sterile saline alone. Following the injections, and at selected time points (1 to 72 h), groups of animals were killed by over exposure to halothane and the inflammatory exudate was withdrawn after washing the peritoneal cavity.

2.4. Leukocyte harvesting and counting

Leukocytes were harvested by washing cavities with 2 ml of saline containing heparin (5 U/ml). Aliquots of the washes were used to determine total cell counts in Neubauer chamber after

dilution in Turk solution (0.2% crystal violet dye in 30% acetic acid). Differential leukocyte counts were performed on stained Hema[3] cell smears.

2.5. Phagocytosis by peritoneal leukocytes

Leukocytes were obtained from the peritoneal cavity of the animals 12 or 48h after i.p. injection of BaV, BjV or saline. Leukocytes (1 x 10^7 cells/ml) were incubated with 2 ml of phosphate-buffered saline (PBS) containing complement-opsonized zymosan for 40 min at 37°C. For opsonization, 2 ml zymosan particles (2,9 mg/ml in PBS) were mixed with 2 ml mice serum and incubated for 30 min at 37°C. The opsonized zymosan particles were then washed, resuspended in PBS at a concentration of 1 mg/ml. The extent of phagocytosis was determined by counting (in a counting chamber) the percentage of cells that had phagocytosed more than four particles of zymosan (Costa Rosa et al., 1994).

2.6. H_2O_2 production by peritoneal leukocytes

The production of H_2O_2 by peritoneal leukocytes was determined in cell suspensions collected from 3 to 48 h after i.p. injections of either the venoms or saline solution. Total cell number was adjusted to 2 x 10^6 cells/ml, centrifuged for 10 min and the cell pellet subsequently resuspended in 1 ml of phenol red solution (PRS; 140 mM NaCl, 10 mM potassium-phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red) containing 8.5 U/ml of horseradish peroxidase, as previously described by Pick and Mizel (1981). Using 96-well flat-bottom tissue culture plates (Corning, USA), 100 µl of the cell suspension were combined with 10 µl of phorbol myristate acetate solution (PMA, 10 ng/well) and incubated at 37°C in a 5% CO_2 atmosphere for 1 h. At the end of the incubation period, 10 µl of 1 N NaOH were added to each

well to stop the reaction. Hydrogen peroxide-dependent phenol red oxidation was spectrophotometrically determined by measurement of absorbance at 620 nm (Titertek Multiscan reader, Labsystems, USA). H_2O_2 concentration values were extrapolated from a standard H_2O_2 calibration curve and expressed as nmoles $H_2O_2/2x10^6$ cells.

2.7. Determination of Nitrite

Release of NO from peritoneal macrophages was evaluated 12, 24, 48 and 72 h after i.p. injection of either the venoms or saline solution. Peritoneal cells were collected in 3 ml saline, centrifuged for 6 min at 500 *g* and the cell pellet was subsequently resuspended in RPMI medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 10% heat-inactivated fetal calf serum and plated onto 96 flat-bottom tissue culture plates (2 x 10^4 cell/well). After 2h incubation at 37°C in a 5% CO_2 atmosphere, the wells were washed three times with RPMI medium to remove non-adherent cells and 100 μl of fresh RPMI were added. The cells were incubated during 48 h at 37° C under a 5% CO_2 atmosphere and at the end of this period, the release of NO from macrophages was determined by the accumulation of nitrite (NO_2^-) in the cell culture supernatants which was measured by the Griess reaction (Green et al., 1982). Aliquots of cell supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethynedianime dihydrochloride in 2.5% phosphoric acid), incubated during 10 min at room temperature and the resulting absorbance was measured at 550 nm. Nitrite concentration values were extrapolated from a calibration curve constructed using sodium nitrite as standard and were expressed as μM NO_2^-.

2.7.1. Drug treatments

In order to pharmacologically characterize the NOS responsible for the BaV and BjV-induced production of NO, selected groups of mice were treated with the NOS inhibitor N^g-nitro-L arginine-methil ester (L-NAME) 50 mg/kg, i.p., 24h and 30 min before and 12h after injection of BaV or BjV, or with aminoguanidine 50 mg/kg i.p., 24h and 100 mg/kg i.p. 30 min before and 8h after injection of the venoms. Control groups of animals received saline injection following the same treatment protocols used for L-NAME or aminoguanidine, respectively.

2.8. Effects of venoms on IFN-γ levels

Interferon gamma (IFN-γ) concentrations in peritoneal exudates were quantified by using an antibody-capture enzyme immunoassay (EIA) technique. Briefly, 96-well plates were precoated with monoclonal antibody XMG 1.2 (5μg/ml, 50 μl/well). Fifty microliters of the samples were added to each well and incubated for 60 min at 37°C. A biotinylated AN18 anti-IFN-γ (5μg/ml, 50 μl/well) antiserum was then added. After 1 h incubation and washing, 50μl of avidin-peroxidase were added, followed by incubation for 45 min at room temperature and addition of the substrate (300 μg/ml of 2,2′-azino-bis ethylbenzthiazoline-6-sulfonic acid). Absorbance was measured at 405 nm and IFN-γ levels were calculated from a calibration curve using recombinant murine IFN-γ as standard.

2.9. Western blot analysis

The presence of iNOS and proteins containing nitrotyrosine residues (NT) in macrophages obtained 6, 12, 24 and 48 h after i.p. injection of either the venoms or saline solution (control) was detected by Western blotting. Briefly, after sodium dodecyl sulfate–polyacrilamide gel

electrophoresis (SDS-PAGE) of total cell lysate proteins (a 7% total polyacrilamide concentration was used for iNOS and 10% for NT), the bands were electro-transferred to nitrocellulose membranes (Bio-Rad, USA), and following blockade of non-specific sites with casein, the membranes were incubated overnight at 4°C with the monoclonal antibodies anti-iNOS (250 ng/ml) or anti-NT (500 ng/ml). An alkaline phosphatase-conjugated rabbit-anti mouse IgG (1:3000; Bio Rad) was used as a secondary antibody and the immunoreactive bands were visualized using a chemiluminiscence detection system (Immun-Star, Bio-Rad). The molecular weights of the immunoreactive bands were estimated from their relative mobility, and their intensity was calculated by densitometric analysis. Nitrotyrosine-containing bovine serum albumin (nitro-BSA) was prepared according to the method described by Beckman et al. (1994) and was included as a positive control in the Western blots for NT.

2.10. Drugs and reagents

N^g-nitro-L arginine-methyl ester (L-NAME), aminoguanidine, phenol red solution and PMA were purchased from Sigma Chemical Co. (MO, USA). Anti-IFN-γ (clones: XMG 1.2 and AN18) and rIFN-γ were purchased from Pharmingen (CA, USA). Anti-iNOS was purchased from Transduction Laboratories (USA) and anti-NT purchased from Upstate Biotechnology Inc.. Rabbit anti mouse IgG and 2,2´-azino-bis (3 ethybenzoline-6-sulfonic acid) were purchased from Bio Rad and Southern Biothecnology Associates Inc (AL, USA), respectively. Hema^3 was obtained from Biochemical Sci. Inc., USA. All others chemicals were of the highest purity commercially available.

2.11. Statistical analysis

Results are expressed as mean ± SEM. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by either Turkey or Student *t*-test. Values of probability less than 5% ($P<0.05$) were considered significant.

3. Results

3.1. Cellular influx into peritoneal cavity

Fig. 1 shows the time-course of leukocyte influx into the peritoneal cavities after injection of *Bothrops* venoms or saline solution. Both venoms evoked a significant cell accumulation in the local of injection. BaV caused an increase in the number of total leukocytes from the 3rd up to 48th h, while BjV-induced leukocyte accumulation was significant from 6th up to 48th h following the injection. For both venoms, leukocyte accumulation returned to the control levels after 72 h (panel A). Differential cell counts showed that cells present in the peritoneal cavity after either BaV or BjV injection were predominantly polymorphonuclear leukocytes (PMN), mainly neutrophils. Maximal neutrophil migration was observed at 6 h and 12 h for BaV and BjV, respectively (panel B). As seen in panel C, 48 h after the injection of any of the venoms, a significant accumulation of mononuclear leukocytes (MN) occurred.

3.2. Phagocytosis induced by BjV and BaV

Since maximal PMN and MN influx was observed 12 and 48h after BaV and BjV i.p. injection respectively, we evaluated phagocytosis at these time points. Fig. 2 shows that both venoms induced a similar increment in the extent of phagocytosis at the studied time: BjV (21% at 12 h and 31% at 48 h) and BaV (23% at 12 h and 22% at 48 h).

3.3. Production of H_2O_2 induced by BjV and BaV

After the i.p. injection of BjV or BaV (5μg/mouse) or sterile saline (control), harvested leukocytes did not show significant spontaneous release of H_2O_2 (data not shown). However, in

the presence of PMA, BaV increased the H_2O_2 production by leukocytes collected from the 3rd h to 12th h, with a peak at 12 h. BjV administration caused increased H_2O_2 production by leukocytes collected only at the 12th h (Table 1).

3.4. Production of NO by macrophages stimulated by BaV and BjV and the effect of NO synthase inhibitors

A significant increase in the production of NO by macrophages was observed 48 h after injection of any of the venoms when compared to that from cells obtained from animals injected with saline solution alone. Two different NOS inhibitors, L-NAME and aminoguanidine, were tested for their capacity to block NO production. As shown in Fig. 3, treatment of the animals with either of these inhibitors, at doses reported in the literature, caused significant decrease in nitrite accumulation in the supernatants of cultured macrophages collected 48 h after the injection of either BaV or BjV.

3.5. Release of IFN-γ in the peritoneal cavity induced by BjV and BaV

The release of IFN-γ was evaluated by EIA in the mouse peritoneal cavity at 6, 12, 24 and 48h after injection of BaV and BjV. A marked increase in IFN-γ levels was observed 48h after i.p. injection of BaV (92% increase) and BjV (90% increase), as compared with animals that received saline solution (Table 2).

3.6. iNOS expression

To better understand the ability of the venoms to induce NO, the expression of iNOS from peritoneal macrophages was analysed by western blot. Macrophages harvested 12h after

injection of the venoms showed augmented expression of iNOS when compared with cells harvested at the 24 h time point (Fig. 4). Cells harvested 48h after the injection of any of the tested venoms did not show detectable immunoreactivity towards iNOS (not shown).

3.7. Presence of nitrotyrosine-containing proteins in macrophages homogenates

The analysis of nitrotyrosine-containing proteins by Western blot of macrophage homogenates revealed a marked increase of immunoreactivity in macrophages obtained 12, 24 or 48 h after the injection of the venoms when compared to cells obtained from control animals (Fig. 5).

4. Discussion

Our results demonstrate that the intraperitoneal injection of 5μg of either BaV or BjV to mice resulted in a time-related inflammatory response. Analysis of the cellular component of this reaction showed that both venoms caused a long lasting leukocyte migration into the peritoneal cavity (3 to 48h). Neutrophils were the predominant cells accumulating during the early stages (3-24h), while mononuclear leukocytes appeared at a later time-point (48h) of the reaction. These results are in agreement with others from the literature showing a marked infiltration of leukocytes into the local of tissue damage after injection of *Bothrops* snake venoms (Gutiérrez et al., 1986; Flores et al., 1993; Lomonte et al., 1993; Acosta De Péres et al., 1996; Farsky et al., 1997).

Leukocytes play a central role in the host defense against offending agents owing to their capacity to produce a variety of inflammatory mediators as well as to present antigens and to ingest and kill microorganisms (Zheng et al., 1999). Moreover, by removing necrotic material, leukocytes set the stage for tissue repair and regeneration. Phagocytosis by leukocytes occurs through activation of a restricted number of membrane receptors: mannose receptor, that recognizes carbohydrates; complement receptor (CR) with specificity for C3b and C3bi fragments from the complement system, and Fc receptor (FcγR) that binds IgG-opsonized particles (Lennartz, 1998; Aderem and Underhill, 1999).

In this study the effects of BaV and BjV on peritoneal leukocyte phagocytic capacity were investigated. Our results showed that BaV and BjV increased the engulfment of zymosan particles by both peritoneal polymorphonuclear (PMN) and mononuclear (MN) cells, with the involvement of complement receptors. In contrast to our results, Souza e Silva et al. (1996) showed that the venom of the South American rattlesnake *Crotalus durissus terrificus* reduces the

capacity of spreading and phagocytosis by peritoneal MN. Such discrepancies indicate that different venoms induce different patterns of inflammation reaction. It is well known that *Bothrops* venoms are strongly pro-inflammatory, whereas the venom of *C. d. terrificus* exerts antiinflammatory effects instead (Giorgi et al., 1993; Landucci et al., 1995).

Since an augmented phagocytic capacity is characteristic of activated inflammatory cells (Costa Rosa, et al., 1994), we investigated the effect of both *Bothrops* venoms on the generation of H_2O_2 and NO by migrating leukocytes. Our results showed that *B. asper* and *B. jararaca* venoms increased the production of H_2O_2 by peritoneal leukocytes, indicating that they are capable of priming leukocytes for the respiratory burst. The effects of BaV on this microbicidal mechanism were longer-lasting than those of BjV, thus suggesting that the components responsible for the induction of this cellular response must be different between both venoms. Taking into account that the maximal production of H_2O_2 occurs at a time-point (12 h) at which a large accumulation of neutrophils is observed in the peritoneal cavity, we assume that these cells may be the main source of H_2O_2 secondary to the stimulation by the venoms. Mononuclear cells appear not to play a major role in this response because increased levels of H_2O_2 were no longer observed at the time of maximum cell accumulation (48h). To our knowledge this is the first report demonstrating the ability of snake venoms to activate the respiratory burst in leukocytes *in vivo*. Although the mechanisms whereby these venoms activate the respiratory burst and induce the release of H_2O_2 are currently unknown, it is suggested that stimulation of phagocytosis may be the first step of this phenomenon.

The influence of the venoms on the release of NO by peritoneal macrophages was also studied. Administration of BjV and BaV induced a significant increase of nitrite (a NO end-product) concentrations in the supernatants of cultured macrophages harvested from mouse peritoneal cavities 48h after the venom injection. This effect was inhibited by pre-treating

animals with either L-NAME (a non-selective NOS inhibitor) or aminoguanidine (at dose proven to be selective for iNOS). These findings implicate iNOS in the production of NO by macrophages secondary to the venom stimulation.

Strengthening the involvement of iNOS in the venom-induced NO release by peritoneal macrophages, we show significant expression of this enzyme in macrophages harvested 12 and 24h following the injection of either BaV or BjV. Interestingly, no significant expression of iNOS was detected in macrophages harvested 48h after injection of these venoms. This apparent lack of correlation between the degree of enzyme expression and the increase in NO production may reflect the lack of sensitivity of the immunoblotting technique.

Gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) have been identified as the key cytokines responsible for induction of iNOS (Ding et al., 1988). To further elucidate the mechanism(s) involved in macrophage iNOS expression secundary to the venom administration, we decided to analyse the release of IFN-γ into the peritoneal cavity. Results showed that i.p. injection of BaV and BjV induced a significant increment in intraperitoneal concentrations of IFN-γ. We have previously shown that the intravenous administration of either BaV or BjV into mice results in a marked increase in serum IFN-γ levels, among other cytokines (Petricevich et al., 2000). Taken together, these results strongly suggest a role for IFN-γ as a mediator of macrophage iNOS synthesis induction by the venoms. However, an alternative direct effect of the venoms on macrophages can not be ruled out.

The actual role that NO plays in the inflammatory reaction associated with *Bothrops* venom injection is not presently known. NO is a short-lived free radical that mediates several physiological functions including neurotransmission (Bredt and Snyder, 1992; Downen et al., 1999) and vasodilation (Palmer et al., 1987). However, in response to inflammatory mediators,

phagocytic cells produce a large amount of iNOS-derived NO (Green et al., 1990; Moncada and Palmer, 1991; Bertholet et al., 1999) which could account for its tumoricidal (Hibbs et al., 1989) and microbicidal activities (Mauël et al., 1991).

NO and superoxide anion, when locally produced at the site of inflammation, can react to form peroxynitrite anions ($ONOO^-$) (Beckman et al., 1990) . Peroxynitrite is not a free radical but a short-lived and far more reactive species than its precursors. It can react with and modify many important biological targets (such as reduced thiol groups, lipids, proteins, and nucleic acids) by different molecular mechanisms (Pryor and Squadrito, 1995) and has been described in a number of pathological inflammatory conditions (Rohn et al., 1999). The nitration of protein tyrosine residues (to form the 3-nitrotyrosine derivative) has been shown to be a marker of peroxynitrite production both *in vivo* and *in vitro* (Beckman et al., 1994). In this work, we show that nitrotyrosine-modified proteins are present in peritoneal macrophages harvested 12 to 48 h after either BaV or BjV injections. The initiation of $ONOO^-$ formation correlates well with the activation of the burst of oxygen consumption and with iNOS expression, indicating a close relationship between these venom-induced reactions. However, these results do not allow us to determine if the $ONOO^-$ detected in macrophages arises from neighboring polymorphonuclear cells or if macrophages themselves are responsible for its formation. It is therefore possible that $ONOO^-$ plays a role in the venom-induced toxic effects, a hypothesis that needs to be addressed in future studies.

Taking the above considerations together, we can hypothetise that NO participates of the pathogenesis of envenomation in distinct ways: (a) it can induce tissue damage due to its ability to form peroxynitrite after reaction with locally generated superoxide anions and, (b) it can contribute to the characteristic hypotension observed in these envenomations.

In conclusion, the present study provides the first experimental evidence that *B. asper* and *B. jararaca* venoms trigger several leukocyte microbicidal functions *in vivo*. Both venoms are able to induce phagocytosis and the release of reactive oxygen species, iNOS-derived NO and IFN-γ. The presence of nitrotyrosine-containg proteins suggests that peroxynitrite can also be involved in the physiopathology of *Bothrops* envenomation. If this is the case, specific iNOS inhibition and/or peroxynitrite scavengers should be considered as potentially useful therapeutical approach for treatment of the local effects of *Bothrops* snakebites.

Acknowledgements

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Fig. 1- Time-course of leukocyte influx into the mouse peritoneal cavity induced by *B. asper* or *B. jararaca* venoms. The venoms of BaV or BjV (5μg/mouse) or sterile saline (control group) were injected i.p.. Animals were anesthetised and the peritoneal cavity washed with PBS for counting cells counts at the time intervals indicated above. Panel A represents the number of total leukocytes, Panel B the number of polymorfonuclear cells (PMN) and panel C the number of mononuclear cells (MN). Each point represents the mean ± S.E.M. from 5-8 animals. *$P< 0.05$ compared with the control group.

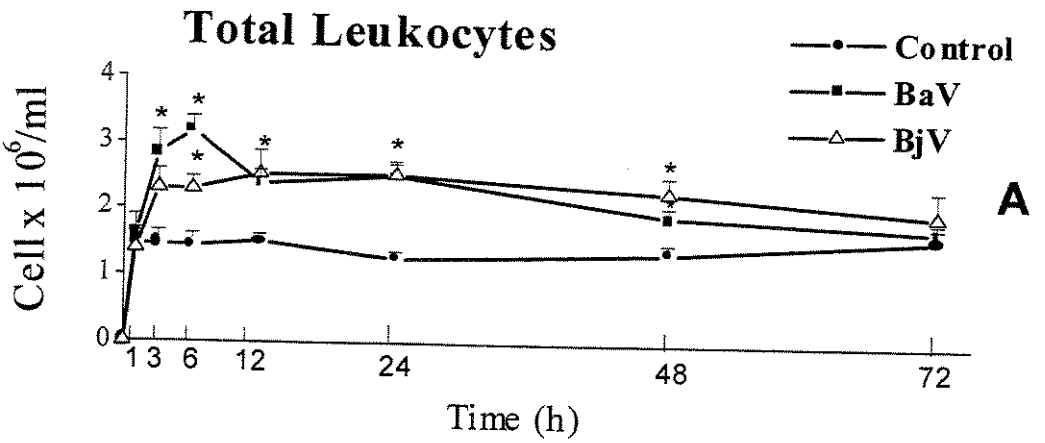
Fig. 2 – Leukocyte mediated phagocytosis of opsonized zymosan particles induced by *B. asper* or *B. jararaca* venoms. Leukocytes were collected from mouse peritoneal cavity 12 and 48 h after i.p. injection of the venoms (5μg/mouse) or sterile saline. $1x10^6$ cells/ml were incubated with PBS containing opsonized zymosan for 40 min at 37°C. The percentage of cells that had phagocytosed more than four particles of zymosan was determined by light microscopic observations. Each value represents the mean ± S.E.M. from 4 animals. * $P< 0.05$ compared with control group.

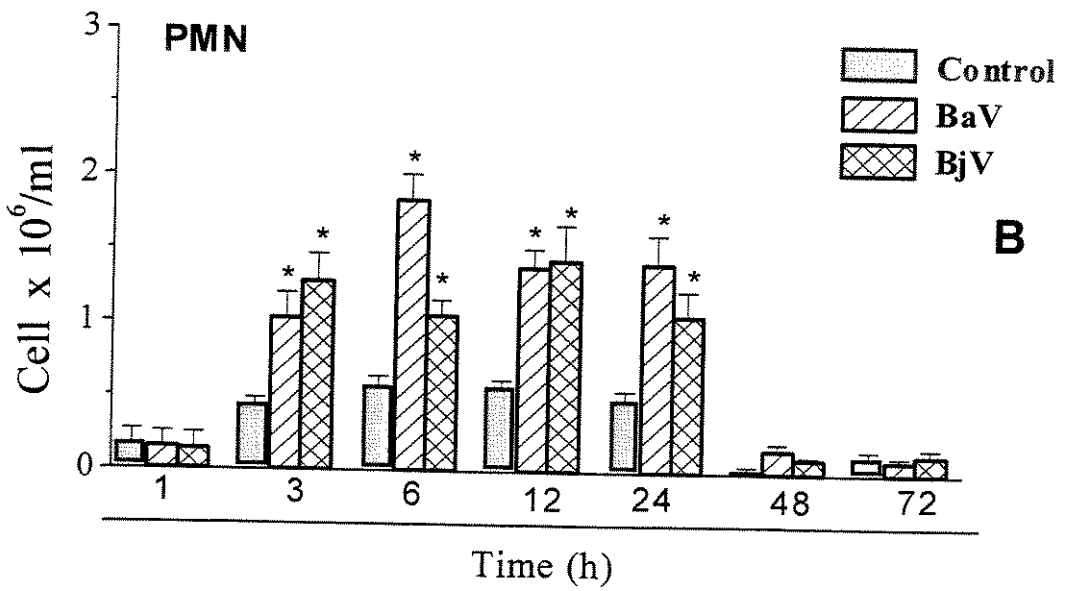
Fig. 3 – Effect of *B. asper* or *B. jararaca* venoms on the NO production by macrophages. Mice were injected i.p. with either BaV or BjV (5μg/mouse) or sterile saline solution (control group). Nitrite concentration in the supernatant from cultured peritoneal macrophages (collected 24, 48 and 72 hours after injections of the venoms) were measured by the Griess reaction. Selected groups of animals were treated with L-NAME or aminoguanidine (AG) (at doses described in Matherial and Methods) before injection of venoms. Data are expressed as mean ±

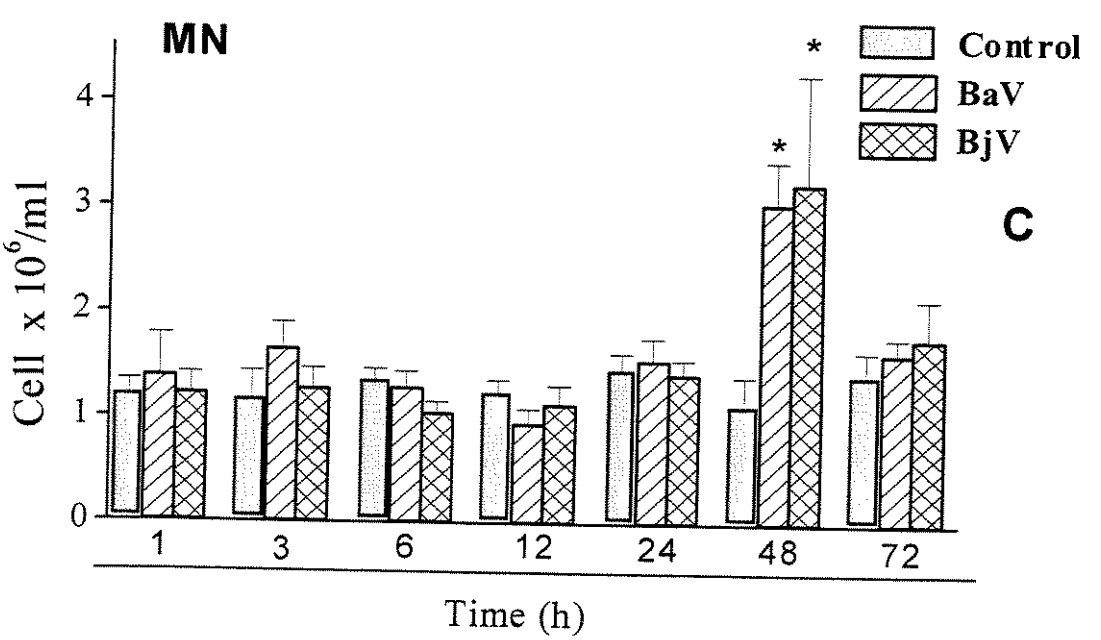
S.E.M. from 8-10 animals. *$P<$ 0.05 as compared to saline (control) value; # P <0.05 as compared to the corresponding venom treated group.

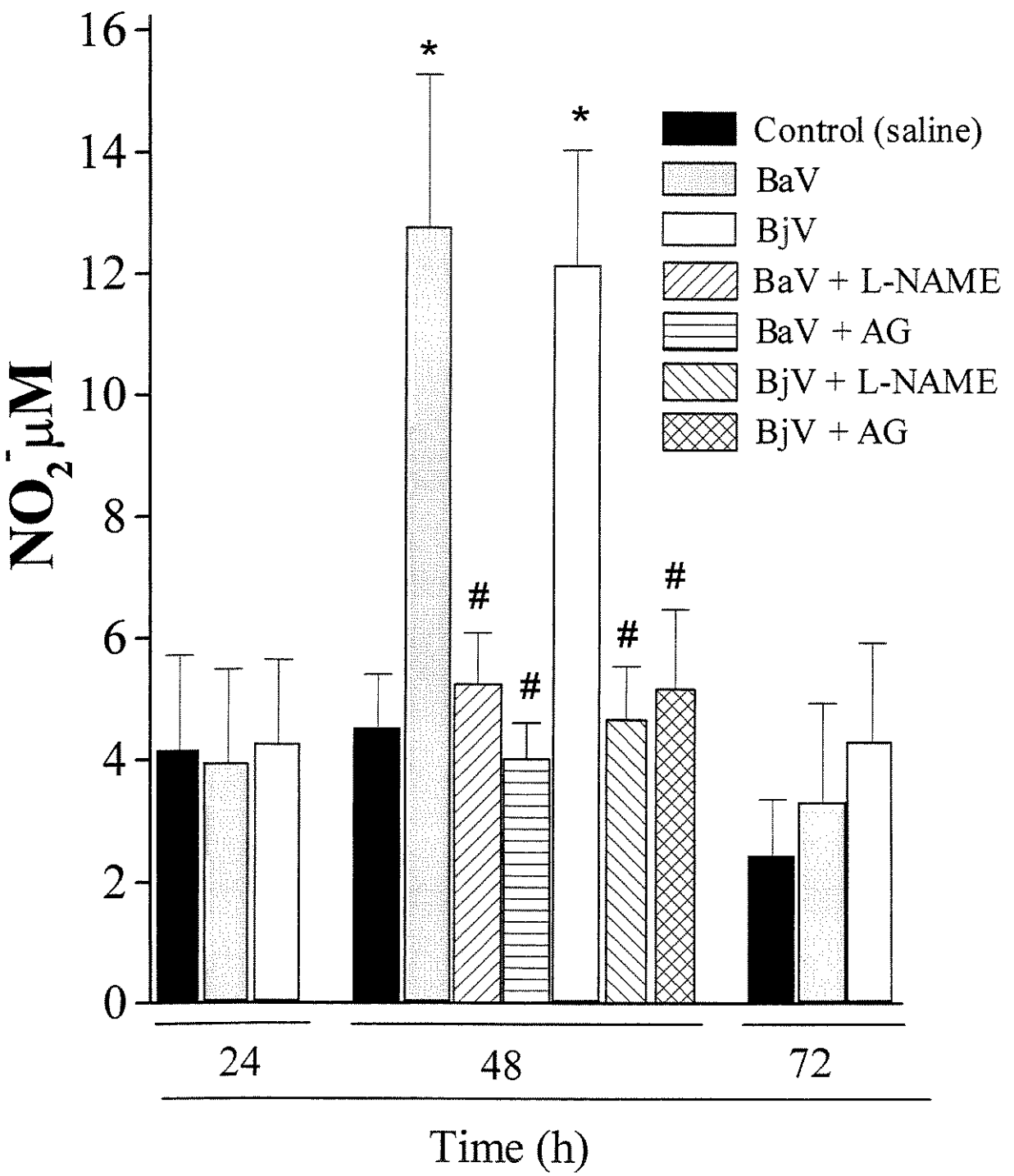
Fig. 4- The effect of *B. asper* and *B. jararaca* venoms on iNOS expression by macrophages. Peritoneal macrophages were harvested 48h after i.p. injection of either BaV or BjV. Whole-cell lysates were obtained and subjected to electrophoresis and Western blot analysis with an anti-iNOS polyclonal antibody, as described in Materials and Methods. (A) Representative blot from three independent experiments. Lane **C**: control; **J**: *B. jararaca* venom; **A**: *B. asper* venom; (+): positive control. (B) Densitometric analysis of immunoreactive band intensities. Results are expressed as mean ± S.E.M. from three experiments. *$P<$ 0.05 as compared to corresponding control value.

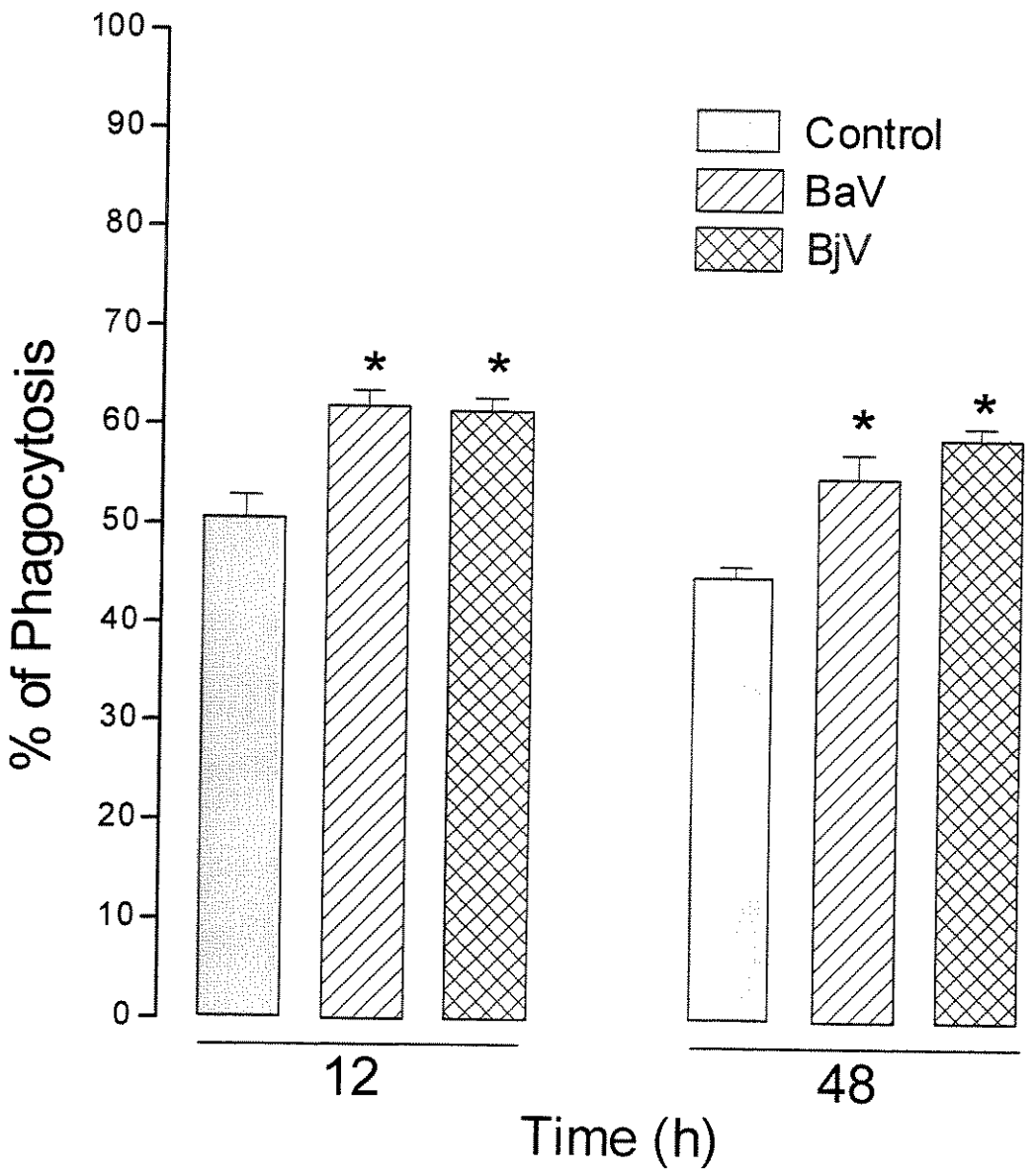
Fig. 5 - Effect of *B. asper* and *B. jararaca* venoms on the expression of nitrotyrosine containing proteins by peritoneal macrophages. Peritoneal macrophages were harvested 12, 24 and 48h after i.p. injection of BaV or BjV. Western blot analysis of the whole cell lysates was performed for nitrotyrosine containing proteins (as described in Material and Methods). The shown immunoblots are representative of four independent experiments. Lane **C**: control; **A**: *B. asper* venom; **J**: *B. jararaca* venom; (NT-BSA): positive control.


Total Leukocytes
Control
BaV
BjV
A
Cell x 10^6/ml
Time (h)
1 3 6 12 24 48 72

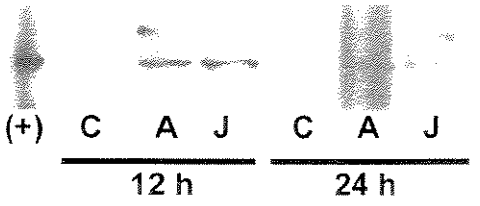

PMN
Control
BaV
BjV
B
Cell x 10^6/ml
Time (h)
1 3 6 12 24 48 72


MN
Control
BaV
BjV
C
Cell x 10^6/ml
Time (h)
1 3 6 12 24 48 72

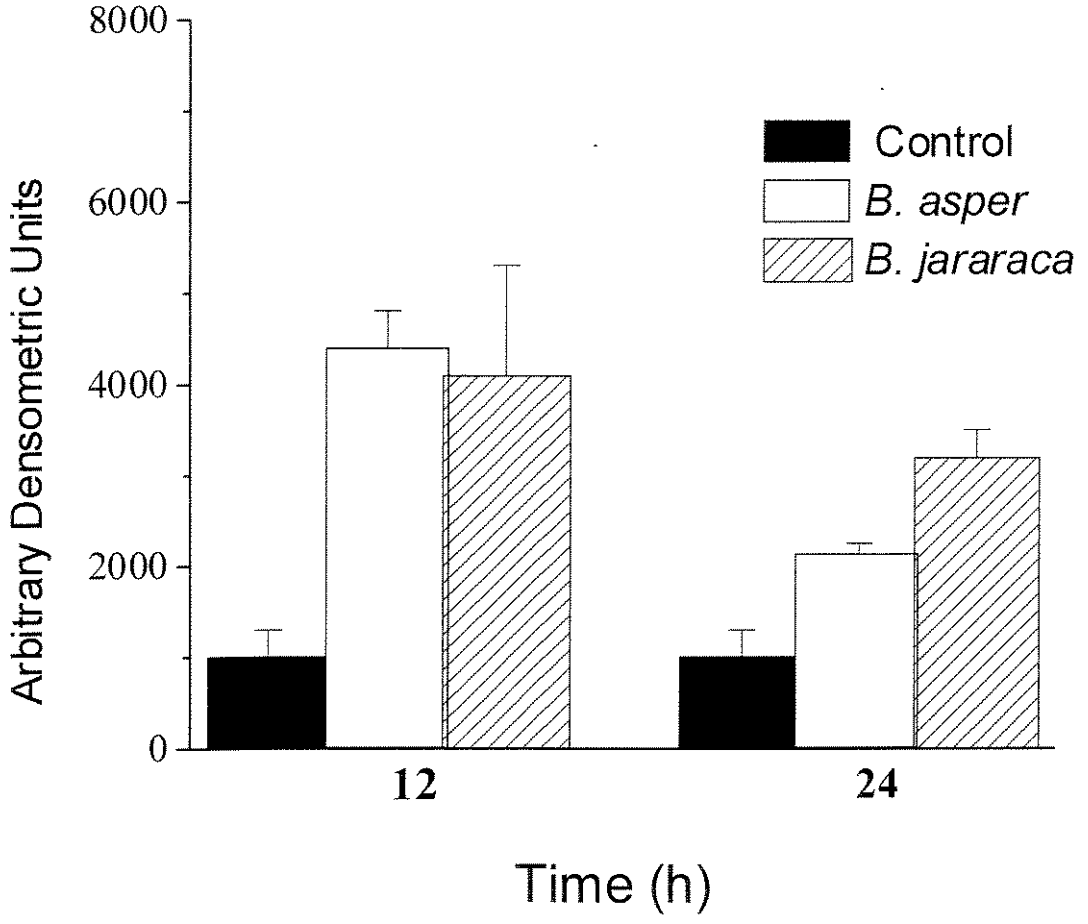

16
14
12
10
8
6
4
2
0
NO_2^- µM
*
*
#
#
#
#
Control (saline)
BaV
BjV
BaV + L-NAME
BaV + AG
BjV + L-NAME
BjV + AG
24
48
72
Time (h)


100
90
80
70
60
50
40
30
20
10
0
% of Phagocytosis
Control
BaV
BjV
*
*
*
*
12
48
Time (h)

A-


(+) C A J C A J
12 h
24 h

B-


8000
6000
4000
2000
0
Arbitrary Densometric Units
Control
B. asper
B. jararaca
12
24
Time (h)

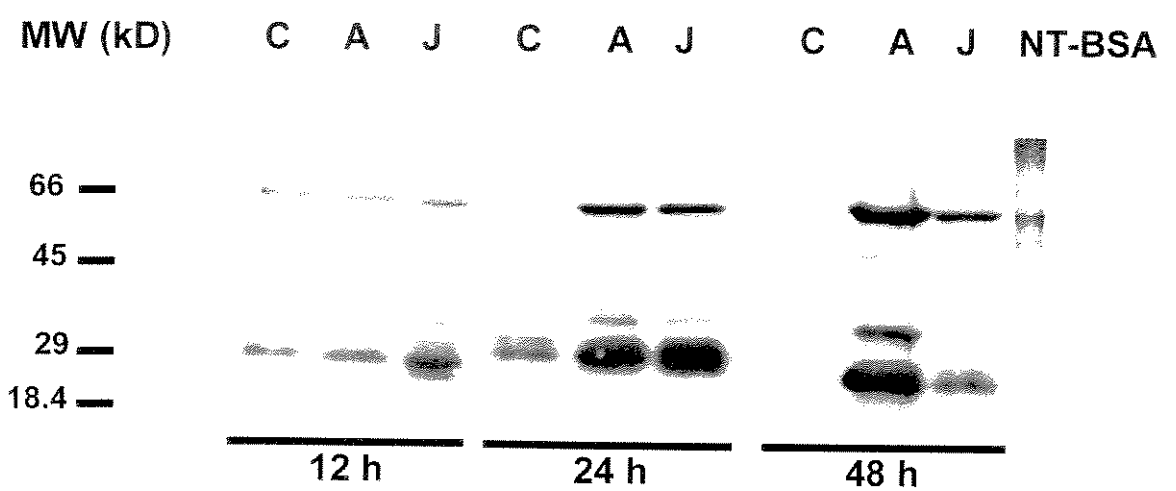

MW (kD)
C A J C A J C A J NT-BSA
66
45
29
18.4
12 h
24 h
48 h

TABLE 1 – Release of H_2O_2 by peritoneal leukocytes induced by either *Bothrops asper* (BaV) or *B. jararaca* (BjV) venoms.

	nmoles H_2O_2 / $2x10^6$ cells		
Time after i.p. injection (h)	Control	BaV	BjV
3	0.40 ± 0.06	0.91 ± 0.10*	0.63 ± 0.20
6	0.49 ± 0.08	0.87 ± 0.12*	0.54 ± 0.07
12	0.35 ± 0.08	1.88 ± 0.19*$^{\Delta}$	1.10 ± 0.19*
24	0.36 ± 0.19	0.59 ± 0.08	0.46 ± 0.08
48	0.18 ± 0.10	0.14 ± 0.04	0.27 ± 0.07

The animals were injected i.p. with *B. asper*, *B. jararaca* venoms (5μg/mouse) or sterile saline solution (control). Peritoneal cells were harvested and $2x10^6$ cells were resuspended in phenol red solution containing horseradish peroxidase (8,5 U/ml). Production of H_2O_2 in the presence of PMA was evaluated 3, 6, 12, 24 and 48 hours after injection of the venoms. Each value represents the mean ± S.E.M. from 4 – 8 animals.

* $P< 0.01$ compared with control. Δ $P< 0.05$ compared with BjV.

TABLE 2 – Release of IFN-γ induced by *Bothrops asper* (BaV) or *B. jararaca* (BjV) venoms in the peritoneal cavity of mice.

	ng IFN-γ/ml		
Time after i.p. injection (h)	Control	BaV	BjV
6	0.12 ± 0,05	0.22 ± 0.13	0.40 ± 0.16
12	0.38 ± 0.09	0.24 ± 0.12	0.37 ± 0.10
24	0.12 ± 0.07	0.13 ± 0.05	0.13 ± 0.07
48	0.05 ± 0.05	0.61 ± 0.10*	0.51 ± 0.11*

The animals were injected i.p. with *B. asper*, *B. jararaca* venoms (5μg/mouse) or sterile saline solution (control). Concentrations of IFN-γ in the peritoneal washes collected 6, 12, 24 and 48 hours after the injection of venoms were measured by EIA. Each value represents the mean ± S.E.M. from 4 animals. * $P < 0.05$ compared with control.

May 02nd, 2001.

D.H.G. Versteeg, Ph.D.
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The Netherlands

Ref. MS E16540 entitled "Effect of long-term nitric oxide synthesis inhibition on the contractile responses in the rat ileum" by Bricola et al.

Dear Dr Versteeg,

Please find enclosed two copies of the above-mentioned MS, together with two sets of figures, our replies to referees (*see doc attached*) and a matched diskette of our revised MS (*labeled as "Bricola_MS_E16540.doc"*).

Corrections of our revised MS were also done according the checklist indications.

We hope you find our MS suitable to be published in European Journal of Pharmacology.

Thank you very much for your attention.

I look forward to hearing from you in due course.

Yours sincerely

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Effect of long-term nitric oxide synthesis inhibition on the contractile responses in the rat ileum

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Abstract

The responsiveness of muscarinic and tackykinin agonists in the intestinal smooth muscle of animals under long-term nitric oxide (NO) synthase blockade has not been studied. The aim of this study was to examine the effects of long-term treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the rat ileum contractions induced by methacholine, carbachol and substance P. Male Wistar rats received L-NAME (20 mg/rat/day) in the drinking water for 7, 15, 30 and 60 days. Control animals received either tap water alone or D-NAME. Concentration-responses curves to methacholine, carbachol and substance P were obtained and pEC_{50} values were calculated. The ileum NO synthase activity was markedly reduced in all L-NAME-treated rats. The potency of methacholine was not significantly affected at 7, 15 and 30 days of L-NAME treatment. However, at 60 days a 4-fold increase in methacholine potency was observed in L-NAME-treated rats (7.16 ± 0.06) compared to control animals (6.56 ± 0.05) or D-NAME group (6.81 ± 0.11). At 60 days, a 3-fold increase in carbachol potency was also observed in L-NAME-treated rats. The supersensitivity to methacholine at 60 days was not modified by L-arginine (1 mM), tetrodotoxin (0.3 μM), indomethacin (3 μM) or 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ; 3 μM). The potency and maximal responses of substance P were not affected by L-NAME treatment. No morphological alterations in the rat ileum were observed in L-NAME-treated rats. In conclusion, long-term NO blockade leads to an increase of the potency for muscarinic agonists in the rat isolated ileum, possibly by functional alterations occurring at post-junctional level.

Keywords: Muscarinic receptors; Tachykinin receptors; N^{ω}-nitro-L-arginine methyl ester; Nitric oxide synthase; Non-adrenergic non-cholinergic fibers.

1. Introduction

The enteric nervous system is composed of the myenteric and the submucosal plexus. The former lies between the longitudinal and circular muscular layers, and controls the gastrointestinal motility whereas the latter lies in the submucosa, and regulates epithelial secretion and local blood flow (Furness et al., 1992). Besides sympathetic and parasympathetic fibers, non-adrenergic non-cholinergic (NANC) nerves exert an important role in the gastrointestinal functions (Bennett, 1997). The NANC innervation consists of two components, namely inhibitory and excitatory. The inhibitory neurons contain ATP (Burnstock, 1990) and vasoactive intestinal peptide (VIP, Fahrenkrug et al., 1978). Additionally, nitric oxide (NO) has been considered the primary inhibitory neurotransmitter in gastrointestinal tissues from a number of animal species as evidenced by both functional (De Man et al., 1991; Kanada et al., 1992; Sanders and Ward, 1992; Suzuki et al., 1994) and immunohistochemical studies (Bredt et al., 1990; Donat et al., 1999). Interactions between the main neurotransmitter of parasympathetic fibers, acetylcholine, and NANC inhibitory fibers have been reported. Acetylcholine can release NO in the gut by activation of pre-synaptic M_1 receptor on myenteric neurons showing that NANC nitrergic and cholinergic fibers interact on the intestinal smooth muscle controlling its motility and neuronal activity (Iversen et al., 1997). Conversely, endogenous NO can inhibit neurotransmission mediated by acetylcholine, probably via a pre-junctional mechanism (Gustafsson et al., 1990; Wilklund et al., 1993). The tachykinins substance P and neurokinin A are the NANC excitatory neurotransmitters in the mammalian intestine (Kunze and Furness, 1999). Furthermore, substance P stimulates the gastrointestinal tract not only by a direct effect on

the smooth muscle, but also through activation of tachykinin NK_3 receptors at pre-synaptic level causing the release of acetylcholine from parasympathetic terminals (Holzer and Holzer-Petsche, 1997). Although considered as an excitatory agent, substance P can also inhibit peristalsis through activation of pre-junctional tachykinin NK_1 receptors present in the inhibitory fibers causing NO release (Holzer, 1997). This interaction has been supported by immunocytochemical studies showing a colocalization of tachykinin NK_1 receptors and NO synthase in the enteric neurons of the guinea-pig gut (Portbury et al., 1996). In addition, endogenous NO has been shown to counteract the actions of substance P. The functional role of these multiple neurotransmitters in the gastrointestinal tract and their interactions with each other is still unclear. Besides, no investigations have been performed to study the responsiveness of muscarinic and tackykinin agonists in the intestinal smooth muscle in animals under long-term blockade of the NO synthase. Therefore, the aim of this study was to examine the effect of long-term administration of a NO synthesis inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), on the contractility of rat isolated ileum induced by muscarinic agonists (methacholine and carbachol) and substance P.

2. Materials and methods

2.1. Animals

Experiments were carried out in male Wistar rats weighing approximately 150 g provided by the Animal Care of State University of Campinas (UNICAMP). All procedures were designed in accordance with the guidelines for animal care of UNICAMP.

2.2. Long-term treatment with L-NAME and blood pressure measurements

The NO synthase inhibitor L-NAME was dissolved in the drinking water at a concentration of 400 mg L^{-1} to give a daily intake of approximately 20 mg/rat/day for 7, 15, 30 or 60 days. The inactive enantiomer D-NAME (20 mg/rat/day) was given only for sixty days. Matched-control groups received tap water alone. The arterial blood pressure was measured weekly by a modified tail-cuff method.

2.3. Tissue preparation

The rats were anesthetized with halothane and exsanguinated. Ileum segments were taken 10 cm from the ileocaecal junction and washed with Krebs-Henseleit solution (pH 7.3-7.5) with the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose. Functional and histological studies on the rat isolated ileum were performed.

2.4. Histological analysis

The rat isolated ileum was cut into pieces of 2 cm, opened and fixed immediately in 10% formalin for 24 h. The tissue was then washed in water and embedded in 70% ethyl alcohol, according to conventional methods for light microscopy. Briefly, the ileum pieces were embedded in paraffin and 5 mm sections were stained with hematoxylin-eosin. For the qualitative analysis, the integrity of the mucosa (especially villus), circular and longitudinal muscles were evaluated. For the quantitative analysis, a morphometric determination of thickness of the circular and longitudinal layers was performed.

2.5. Functional assays

Segments of 1.5 cm of the rat ileum obtained at 7, 15, 30 or 60 days after L-NAME treatment or the age-matched control groups were mounted in a 10 ml organ bath containing Krebs solution at 37°C, continuously gassed with a mixture of 5% CO_2 and 95% O_2. A tension of 2 g was applied and the tissues were allowed to equilibrate for 45 min. Cumulative concentration-response curves to methacholine were obtained in absence or in presence of the following drugs: L-arginine (1 mM), tetrodotoxin (0.3 μM), indomethacin (3 μM), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 3 μM) and atropine (1-100 nM) after 30 min incubation. Concentration-response curves to substance P were also obtained in presence of cyproheptadine (1 μM), atropine (10 nM) and tetrodotoxin (0.3 μM) to block indirect actions of substance P (Maggi and Giuliani, 1995). Isotonic contractions were recorded using an isotonic transducer (Harvard Apparatus) and a Watanabe multichannel pen recorder (model WTR 381).

2.6. Construction of concentration-response curves

Cumulative concentration-response curves to methacholine, carbachol and substance P were constructed by using one-half log unit increments (Van Rossum, 1963). All concentration-response data were evaluated for a fit logistic equation:

$$E = E_{max}/((1+(10^{c}/10^{x})^{n}) + \Phi)$$

where E is the increase in rate above basal; E_{max} is the maximum response that the agonist can produce; c is the logarithm of the EC_{50}, the concentration of agonist that produces half-maximal response; x is the logarithm of the concentration of agonist; the exponential term, n, is a curve fitting the parameter that defines the slope of the concentration-response line, and Φ is the response observed in the absence of added agonist. Nonlinear regression analyses to determine the parameters E_{max}, log EC_{50} and n were done using GraphPad Prism (GraphPad Software, San Diego, CA) with the constraint that Φ = zero. The displacement on the cumulative concentration-response curve to methacholine induced by the competitive antagonist atropine was analysed by Schild regression (Arunlakashana and Schild, 1959).

2.7. Assay of ileum constitutive NO synthase activity

The animals were killed with CO_2 asphyxiation and the isolated ileum (10 cm) was dissected and cleaned in physiological saline. The tissue was homogenized in 50 mM Tris, buffer pH 7.4 (100 ml), containing L-citrulline (1 mM), EDTA (0.1 mM), dithiothreitol (1 mM), leupeptin (10 μg ml^{-1}), soybean trypsin inhibitor (10 μg ml^{-1}), aprotinin (2 μg ml^{-1}), phenyl methyl sulfonyl fluoride (1 mM). The tissue homogenization was performed using a Ultra-Turrax

T25 in 5 ml of buffer per g of tissue (wet weight). The homogenates were centrifuged at 12,000 g for 10 min and NO synthase activity was measured in the supernatant fractions according to Lincoln and Messersmith (1995). Briefly, the supernatant fractions (50 µl) were incubated in a modified Tris buffer (50 mM, pH 7.4) containing $CaCl_2$ (1 mM), FAD (10 µM), NADPH (1 mM), calmodulin (10 µg ml^{-1}) and BH_4 (100 µM; Hiki *et al.*, 1992), previously equilibrated at 37^0 C for 5 min. Pharmacological controls of enzymatic activity were carried out in parallel and consisted of either the omission of $CaCl_2$ and addition of EGTA (1 mM) or the addition of L-NAME (1 mM) to the incubation medium. The L-[2,3,4,5-^{3}H]arginine solution was prepared by diluting [^{3}H]arginine with non-labeled L-arginine to give a final [^{3}H]arginine concentration of 10 µM and 200,000 dpm. After 15 min, the reaction was stopped by addition of 1 ml of the ice-cold buffer (pH 5.4) containing HEPES (20 mM), EGTA (1 mM), EDTA (1 mM), followed by vortex mixing. The samples were then applied to a 0.6 ml DOWEX 50wx8-200 (ionic form: hydrogen, dry mesh 100-200) pre-equilibrated with the stopping buffer. L-[2,3,4,5-^{3}H]L-citrulline was eluted and washed with 1 ml of stopping buffer and the radioactivity was determined by liquid scintillation counting. All measurements were made in duplicate. Protein concentrations were determined using bovine serum albumin as a standard. Activity was expressed as pmol of citrulline mg^{-1} protein min^{-1}, and the values were corrected from the remaining NO synthase activity in presence of exogenous L-NAME.

2.8. Drugs

N^{ω}-nitro-L-methyl arginine (L-NAME), D-NAME, carbachol, atropine sulfate salt, tetrodotoxin, indomethacin, substance P, L-arginine hydrocloride, cyproheptadine hydrochloride, methacholine (acetyl-β-methycholine chloride), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one

(ODQ) and all reagents used in determination of ileum NO synthase activity were purchased from Sigma Co (St. Louis, MO). L-[2,3,4,5-^{3}H]arginine (specific activity of 41 Ci/mmol) was supplied by Amersham Pharmacia Biotech (UK).

2.9. Statistical analysis

All values are expressed as the means ± S.E.M. The program InStat (GraphPad Software) was used for statistical analyses. Where appropriate, one-way analyse of variances (ANOVA) followed by a Newman-Keuls multiple comparisons *post hoc* test were performed. In some cases, a paired or unpaired Student's *t* tests were used. $P < 0.05$ was accepted as significant.

3. Results

3.1. Systolic blood pressure

The long-term administration of L-NAME caused a time-dependent and significant ($P < 0.05$) increase in systolic blood pressure, as evaluated at 7, 15, 30 and 60 days (136 ± 1.3, 167 ± 2.3, 172 ± 0.7 and 204 ± 7.9 mmHg, respectively) compared to control animals (122 ± 1.9 mmHg at 60 days of treatment). The inactive enantiomer D-NAME had no effect on systolic blood pressure (118 ± 1.2 mmHg at 60 days of treatment). The average daily intake of both water and food did not differ among the L-NAME, D-NAME and control groups.

3.2. NO synthase activity

Fig. 1 shows that long-term L-NAME treatment markedly reduced the activity of ileum NO synthase at 7, 15, 30 and 60 days after treatment. In absence of Ca^{2+} ions and addition of EGTA (0.1 mM) to the incubation media, the ileum NO synthase activity was inhibited by 80% in control group, confirming that conversion of [^{3}H]arginine to [^{3}H]citrulline was mainly due to a calcium-dependent constitutive NO synthase. Similarly, at 7 days after treatment, omission of Ca^{2+} ions and addition of EGTA significantly inhibited the Ca^{2+}-dependent constitutive NO synthase activity. At 15, 30 and 60 days, no changes were found between L-NAME-treated groups and age-matched groups with omission of Ca^{2+} ions and addition of EGTA.

3.3. Agonist potency and maximal responses to methacholine and carbachol

No significant changes on the concentration-response curves to methacholine were observed at 7, 15 and 30 days after L-NAME treatment, but a 4-fold increase at pEC_{50} level for this muscarinic agonist was observed at 60 days (Fig. 2 and Table 1). Maximal responses to methacholine were not affected by the L-NAME treatment in any studied time (Table 1). Similarly to methacholine, a 3-fold increase ($P<0.05$) at pEC_{50} level was found for carbachol at 60 days after L-NAME treatment (6.69 ± 0.05 and 7.17 ± 0.09 for control and treated animals, respectively; n=6) without affecting the maximal responses (67 ± 3.5 and 78 ± 4.5 mm for control and treated animals, respectively). For further experiments, methacholine was routinely used.

Treatment of animals with the inactive enantiomer D-NAME did not produce any significant displacement of the log concentration-response curves for methacholine, as observed 60 days after treatment (6.81 ± 0.11 and 6.79 ± 0.10, pEC_{50} for control and D-NAME groups, respectively; n=8; Fig. 2).

Addition of L-arginine (1 mM) affected neither pEC_{50} nor maximal responses to methacholine at 60 days after L-NAME treatment (Table 2). Moreover, the cyclooxygenase inhibitor indomethacin (3 μM) had no effect on the potency and maximal responses for methacholine on isolated ileum obtained from control or L-NAME (60 days)-treated rats (Table 2).

3.4. L-NAME and muscarinic receptors

Addition of L-NAME in vitro (0.1-3 mM) on isolated ileum did not produce any significant shift of the concentration-response curves for methacholine (6.64 ± 0.01, 6.81 ± 0.008,

6.80 ± 0.007, 6.74 ± 0.007 and 6.65 ± 0.006, at pEC_{50} values for control and L-NAME 0.1, 0.3, 1 and 3 mM, respectively; n=8).

Atropine (1-100 nM) exhibited a competitive antagonism for methacholine in the isolated rat ileum, as expected. The pA_2 value for atropine was 9.70 ± 0.09 (n=6) and the Schild regression indicated a slope that did not differ significantly from unity [0.92 (0.87-0.97, 95% of confidence interval)].

3.5. Effect of tetrodotoxin

In control rats, tetrodotoxin (0.3 μM) caused a small, but significant ($P < .05$), increase on the potency of methacholine in the isolated ileum whereas the maximal responses were not significantly affected by this compound (Table 3). In L-NAME (60 days)-treated rats, tetrodotoxin did not change the supersensitivity of rat ileum to methacholine, but decreased by 15 % ($P < 0.05$) the maximum response to this muscarinic agonist (Table 3).

3.6. Effect of 1H-[1,2,4]oxadiazolo[4,3-a]-Quinoxalin-1-One (ODQ)

In isolated ileum from control rats, the potency and maximal responses of methacoline were not affected by the addition of the soluble guanylate cyclase inhibitor ODQ (3 μM, n=8; Table 3). Similarly, in tissues obtained from L-NAME (60 days)-treated rats, ODQ (3 μM) failed to affect the increased pEC_{50} values and maximal responses for methacholine (n=6; Table 3).

3.7. Agonist potency and maximum response to substance P

Concentration-response curves for substance P in the isolated ileum were obtained in presence of cyproheptadine (1 μM), atropine (0.01 μM) and tetrodotoxin (1 μM) to avoid indirect contractions evoked by its actions on the myenteric plexus. Our results showed that the potency of substance P in control animals (5.93 ± 0.12, n=6) did not differ from L-NAME (60 days)-treated rats (6.09 ± 0.12, n=6). The maximal responses for substance P were also unaffected by the L-NAME treatment (60.5 ± 5.92 and 73.7 ± 5.27, P = .13, n=6).

3.8. Quantitative and qualitative histological analysis

Treatment of rats with L-NAME for 60 days (n=6) did not cause any morphological alterations in the rat ileum, as evaluated by the measurement of the thickness of either longitudinal (28.9 ± 1.4 and 32.5 ± 3.5 μm for control and treated groups, respectively) or circular layers (54.9 ± 3.2 and 47.0 ± 3.3 μm for control and treated groups, respectively). Regarding to mucosa layer, the L-NAME treatment did not cause any microscopic injury, especially villus (n=11), compared to tissues from age-matched control groups (n=6).

4. Discussion

This study shows that inhibition of ileum constitutive NO synthase activity by long-term treatment (60 days) of rats with the NO inhibitor L-NAME causes a significant increase in the potency of muscarinic agonists, an effect unaltered by addition of L-arginine, indomethacin, tetrodotoxin and ODQ. These findings indicate a modulatory role of NO on the contractile response mediated by muscarinic receptors in isolated ileum. The alterations to methacholine were due to NO inhibition since animals receiving the inactive enantiomer D-NAME did not show any changes in the potency to this muscarinic agonist. Furthermore, the lack of any morphological lesions in the rat ileum after L-NAME treatment supports the concept that the increased potency for methacholine at 60 days reflect functional rather than pathological adaptative alterations.

Our results showed that the ileum constitutive NO synthase activity was similarly reduced by L-NAME at 7, 15, 30 and 60 days post-treatment, but the increased responsiveness (4-fold) to methacholine was observed only at 60 days demonstrating that a long-term NO blockade is necessary to produce the alterations of muscarinic receptor responses. Similarly, a 3-fold leftward shift to carbachol was observed at 60 days. Although our results are still unclear, we could postulate that supersensitivity to muscarinic agonists at 60 days reflects slow onset mechanisms including increases in the efficiency of coupling between agonist-receptor events and/or changes in the membrane environment (i.e., membrane modifying proteins that modulate the binding of agonists to receptors) that would affect the potency of methacholine and carbachol (Kenakin, 1993). We also tested the effect of exogenous L-arginine to revert the supersensitivity for methacholine in L-NAME-treated group. In previous studies, addition of L-arginine prevented

the inhibitory effects of NO synthase inhibitors on the electrical stimulation-induced contractions of rat gastric fundus (Boeckxstaens et al., 1991a), guinea-pig gastric fundus (Lefebvre et al., 1992) and guinea-pig ileum (Williams and Parsons, 1995). In an in vivo study, L-arginine also prevented the increase in rat jejunal motility by acute L-NAME administration (Calignano et al., 1992). In our study no changes were observed by L-arginine addition suggesting that supersensitivity for methacholine in L-NAME-treated rats does not involve NO deficiency but rather reflects a complex interaction between parasympathetic fibers and NANC innervation in rat ileum. In a previous study, L-NAME was reported to antagonize muscarinic receptors in rabbit coronary arteries and canine colonic smooth muscle (Buxton et al., 1993), a data not confirmed in latter investigations (Suzuki et al., 1994; Bartho et al., 1999). Our findings show that addition of L-NAME *in vitro* (up to 10^{-4} M) had no antimuscarinic actions in rat isolated ileum while in a parallel experiment a classical muscarinic receptor antagonist, atropine, exhibited a competitive antagonism in the concentration-response curves to methacholine. Therefore, the leftward displacement of concentration-response curves to methacholine seen in L-NAME-treated animals at 60 days was not attenuated by any potential antimuscarinic actions of L-NAME.

The mechanisms by which NO regulates the tone of the gastrointestinal tract are still a matter of controversy. While some authors postulate that the effects of NO in gastrointestinal muscle are coupled to cGMP production since they can be blocked by the guanylate cyclase inhibitor ODQ (Eklad and Sundler, 1997; Sanders and Keef, 2000), others suggest that a cGMP-independent process and smooth muscle cell hyperpolarization via K^+ channel activation are involved (Koh et al., 1995). It has also been proposed that both cGMP-dependent and independent mechanisms can mediate the NO actions in the gut (Cellek et al., 1996; Bayguinov and Sanders, 1998). Our data that ODQ failed to affect the methacholine-induced contractile

responses rule out the involvement of cGMP as a transductional signal in the rat ileum. This is reinforced by the findings that cGMP levels are unaltered in the guinea-pig ileum stimulated with carbachol (Hartle, 1976).

Various cyclooxygenase products stimulate cholinergic neuro-effector transmission. In guinea-pig ileum, endogenous prostacyclin modulates acetylcholine release from cholinergic nerves terminals in the myenteric plexus (Fukunaga et al, 1993). Additionally, a molecular cross-talk between NO and cyclooxygenase-derived products regulating tissue homeostasis and pathophysiological processes has been reported (Clancy et al., 2000). Thus, endogenous NO (Stadler et al., 1993) or NO-releasing compounds (Kosonen et al., 1998) can inhibit the prostanoid production in various cell types and tissues. However, the possibility that the increased sensitivity to methacholine by long-term L-NAME treatment could be due to cyclooxygenase up-regulation, and consequently an enhancement of cyclooxygenase-derived products was tested by using the cyclooxygenase inhibitor indomethacin. Our findings that indomethacin had no effect on the concentration-response curves for methacholine in control and L-NAME-treated animals show that prostanoids are not involved on the supersensitivity to methacoline by long-term L-NAME treatment.

A bi-directional interaction between acetylcholine and nerve-evoked NO release in the gut has been reported. Thus, exogenous acetylcholine releases NO/NO^{-}_{2} through activation of muscarinic M_1 receptors in the guinea-pig colon (Iversen et al., 1997) and, on the other hand, NO itself inhibits the nerve-evoked acetylcholine release in the guinea-pig ileum (Kilbinger and Wolf, 1994). Therefore, the supersensitivity for methacholine caused by long-term NO blockade might reflect an increased release of acetylcholine from enteric nerves. This hypothesis was tested using tetrodotoxin, a Na^{+} channel blocker that inhibits nerve-mediated responses. Our results showed that in control (untreated) animals, tetrodotoxin increased by approximately 2-fold

the potency for methacholine. A number of studies have reported the importance of NO as an inhibitory neurotransmitter in intestinal smooth muscles (Thornbury et al., 1991; Sanders and Ward, 1992). Since NO generated from gastrointestinal smooth muscle can be inhibited by tetrodotoxin (Boeckxstaens et al., 1991b), our findings suggest that inhibitory pre-synaptic mechanisms can modulate the potency of muscarinic agonists. However, in L-NAME-treated rats, tetrodotoxin did not affect the supersensitivity for methacholine showing that this alteration occurs independently of neural transmitter release, and suggests that a post-junctional event contributes to the overall phenomenon. Therefore, the supersensitivity to methacholine in isolated ileum after L-NAME treatment at 60 days might reflect an increase of muscarinic receptor densities and/or enhancement of receptors coupling events such as G protein, contractile protein and Ca^{2+} mobilization.

In an attempt to evaluate whether the supersensitivity to methacholine in response to long-term L-NAME treatment could also be observed with another excitatory neurotransmitter in the gastrointestinal smooth muscle, the tachykinin substance P was tested. In the musculature of the rat small intestine all three tachykinin receptors are expressed, as evidenced by using selective antagonists and agonists (Hellström et al., 1994) and activation of tachykinin NK_1, NK_2 and NK_3 receptors evokes an increase in the gastrointestinal motility (Willis et al., 1993). Our results showed that the contractile responses of rat ileum to exogenous substance P were unaffected by L-NAME treatment indicating that long-term NO blockade affects the smooth muscle contractility mediated by muscarinic (but not by tachykinin) receptors.

In summary, our findings show an increase in potency for methacholine and carbachol after 60 days of in vivo L-NAME treatment, possibly by mechanisms involving functional alterations of smooth muscle responsiveness mediated by muscarinic receptors at post-junctional level.

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Table 1

Effect of long-term L-NAME treatment on the potency (pEC_{50}) and maximal responses (E_{max}) to methacholine in isolated rat ileum.

GROUPS	**pEC_{50}**	**SHIFT**[a]	**E_{max} (mm)**	**N**
Control 7 days	6.95 ± 0.04		65.7 ± 3.4	9
L-NAME 7 days	7.20 ± 0.09	1.77	67.6 ± 4.2	8
Control 15 days	6.85 ± 0.06		63.4 ± 3.3	11
L-NAME 15 days	7.01 ± 0.05	1.44	72.9 ± 2.6	11
Control 30 days	6.66 ± 0.05		67.3 ± 2.6	15
L-NAME 30 days	6.80 ± 0.04	1.38	74.3 ± 2.0	13
Control 60 days	6.56 ± 0.05		78.4 ± 4.4	9
L-NAME 60 days	7.16 ± 0.06[b]	3.98	67.3 ± 3.4	9

Data are means ± S.E.M. for *N* animals.

[a] Arithmetic multiple for change on potency following L-NAME treatment.

[b] $P < 0.05$ compared to age-matched group.

Table 2

Potency (pEC_{50}) and maximal responses (E_{max}) for methacholine in the isolated ileum obtained from control and L-NAME (60 days)-treated rats in absence or presence of either L-Arginine (1 mM) or Indomethacin (3 μM) *in vitro*.

		L-arginine (1 mM)		Indomethacin (3 μM)	
Groups		Absence	Presence	Absence	Presence
Control	pEC_{50}	6.73 ± 0.08	6.75 ± 0.04	6.74 ± 0.13	6.86 ± 0.10
	E_{max} (mm)	69.2 ± 3.0	73.8 ± 2.9	72.4 ± 4.3	71.1 ± 3.4
L-NAME	pEC_{50}	7.24 ± 0.10[a]	7.08 ± 0.13[a]	7.14 ± 0.15[a]	7.15 ± 0.12[a]
	E_{max} (mm)	65.7 ± 4.0	61.1 ± 3.3	82.2 ± 2.7	79.4 ± 2.9

Data are mean ± S.E.M. for 5-12 experiments.

[a] $P < 0.05$ compared to respective control values.

Table 3

Effect of tetrodotoxin and ODQ on the potency (pEC_{50}) and maximal responses (E_{max}) for methacoline in isolated ileum from control and L-NAME-treated rats for 60 days.

		Tetrodotoxin (0.3 μM)		ODQ (3 μM)	
Groups		Absence	Presence	Absence	Presence
Control	pEC_{50}	6.77 ± 0.10	7.01 ± 0.07[b]	6.97 ± 0.05	7.11 ± 0.07
	E_{max} (mm)	56.3 ± 4.0	53.5 ± 2.4	58.8 ± 3.9	56.3 ± 3.0
L-NAME	PEC_{50}	7.07 ± 0.05[a]	7.11 ± 0.08[a]	7.29 ± 0.11[a]	7.46 ± 0.08[a]
	E_{max} (mm)	71.4 ± 2.5	61.2 ± 3.2[a]	65.6 ± 4.5	61.2 ± 3.8

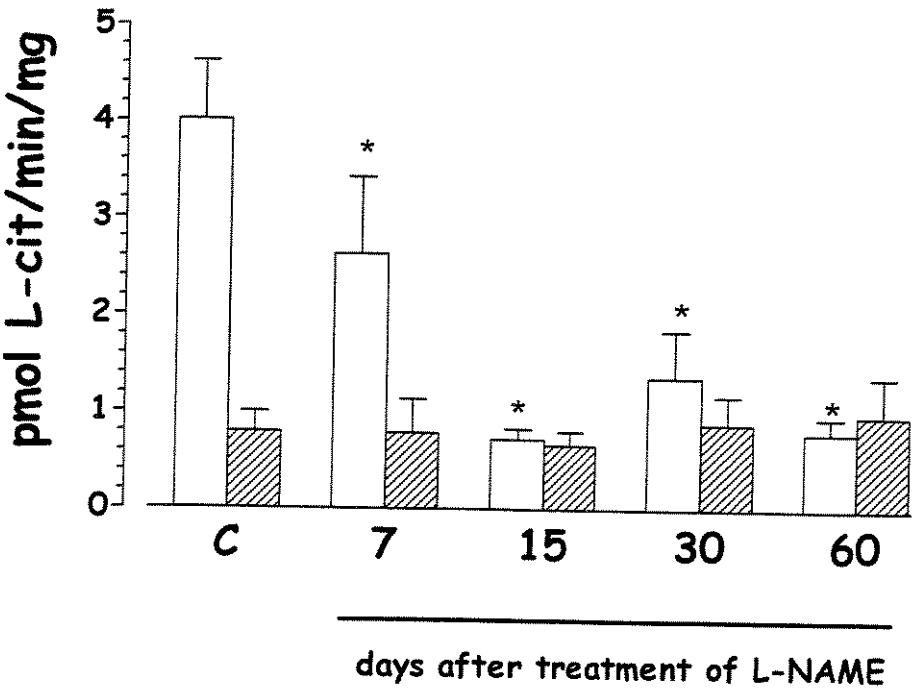
Data are means ± S.E.M. for 6-11 experiments.

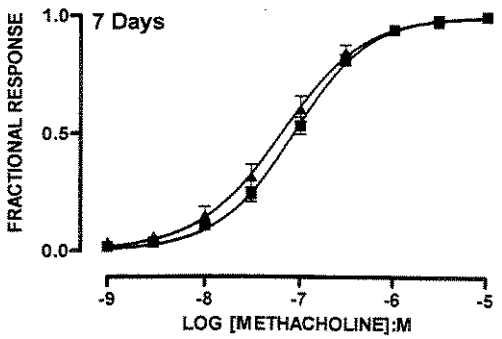
[a] $P < 0.05$ compared to respective control values.

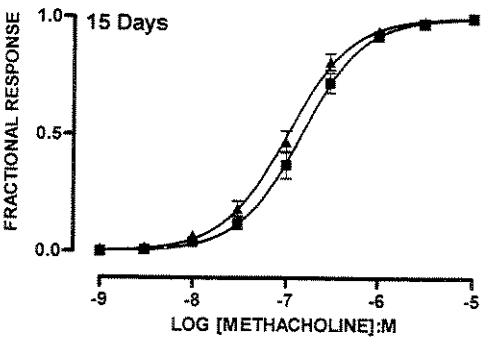
[b] $P < 0.05$ compared to values in absence of tetrodotoxin.

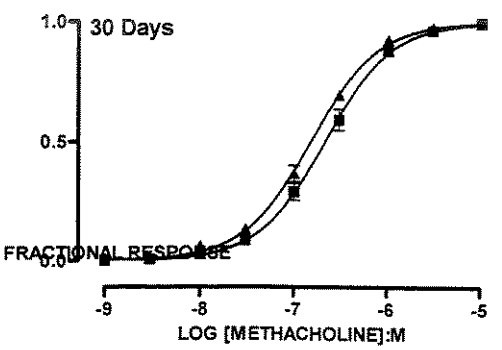
Fig. 1. Nitric oxide (NO) synthase activity in the ileum homogenates from control (C) and L-NAME-treated rats. L-NAME (20 mg/rat/day) was dissolved in drinking water and given for 7, 15, 30 and 60 days, as stated in the *Methods* section. The open bars represent the NO synthase activity under standard conditions for control (C) and L-NAME-treated animals whereas hatched bars represent the activity in the absence of Ca^{+2} ions and in presence of EGTA (1 mM). Activity was expressed as pmol of citrulline mg-1 protein min-1. Data are means ± S.E.M. of 5 experiments. $*P < 0.05$ relative to the control group.

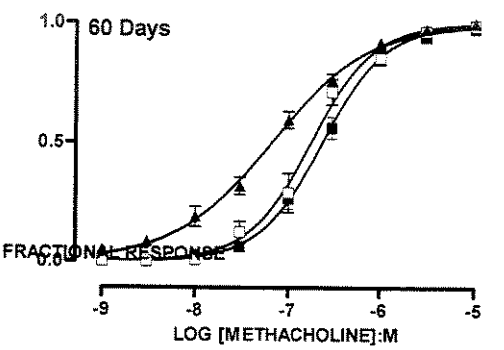
Fig. 2. Concentration-response curves to methacholine in isolated rat ileum from control (■) and L-NAME-treated rats at 7, 15, 30 and 60 days (•). Treatment with D-NAME (□) is showed only after 60 days of treatment. Data are means ± S.E.M. for 8-15 experiments.


pmol L-cit/min/mg
5
4
3
2
1
0
*
*
*
*
C
7
15
30
60
days after treatment of L-NAME


7 Days
FRACTIONAL RESPONSE
1.0
0.5
0.0
-9
-8
-7
-6
-5
LOG [METHACHOLINE]:M


15 Days
FRACTIONAL RESPONSE
1.0
0.5
0.0
-9
-8
-7
-6
-5
LOG [METHACHOLINE]:M


30 Days
FRACTIONAL RESPONSE
1.0
0.5
0.0
-9
-8
-7
-6
-5
LOG [METHACHOLINE]:M


60 Days
FRACTIONAL RESPONSE
1.0
0.5
0.0
-9
-8
-7
-6
-5
LOG [METHACHOLINE]:M