UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE CIÊNCIAS MÉDICAS

CYNTHIA DE MOURA BORGES

NEFROPROTEÇÃO COM EPIGALOCATEQUINA GALATO OU METFORMINA NA DOENÇA RENAL CRÔNICA: UM ENSAIO CLÍNICO E UM ESTUDO EXPERIMENTAL

CAMPINAS

2020

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NEPHROPROTECTION WITH EPIGALLOCATECHIN GALLATE OR METFORMIN IN CHRONIC KIDNEY DISEASE: A CLINICAL TRIAL AND AN EXPERIMENTAL STUDY

Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de doutora em Ciências na área de Clínica Médica.

ORIENTADOR: Prof. Dr. José Butori Lopes de Faria

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA CYNTHIA DE MOURA BORGES E ORIENTADA PELO PROF. DR. JOSÉ BUTORI LOPES DE FARIA.

CAMPINAS

2020

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Médicas
Maristella Soares dos Santos - CRB 8/8402

Borges, Cynthia de Moura, 1976-
B644n Nefroproteção com *epigalocatequina galato* ou metformina na doença renal crônica : um ensaio clínico e um estudo experimental / Cynthia de Moura Borges. – Campinas, SP : [s.n.], 2020.

Orientador: José Butori Lopes de Faria.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Doença renal crônica. 2. Albuminúria. 3. Chá verde. 4. Metformina. 5. Ampk. I. Faria, Jose Butori Lopes de, 1955-. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Nephroprotection with *epigallocatechin gallate* or metformin in chronic kidney disease : a clinical trial and an experimental study
Palavras-chave em inglês:
Chronic kidney disease
Albuminuria
Green tea
Metformin
Ampk
Área de concentração: Clínica Médica
Titulação: Doutora em Ciências
Banca examinadora:
José Butori Lopes de Faria [Orientador]
Lúcia da Conceição Andrade
Márcio Dantas
Rodrigo Bueno de Oliveira
Sara Teresinha Olalla Saad
Data de defesa: 20-10-2020
Programa de Pós-Graduação: Clínica Médica

Identificação e informações acadêmicas do(a) aluno(a)
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- Currículo Lattes do autor: http://lattes.cnpq.br/3654771938919080

COMISSÃO EXAMINADORA DA DEFESA DE DOUTORADO

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Data de Defesa: 20/10/2020

A ata de defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da FCM.

Dedico esta tese aos meus pais, aos meus irmãos, à minha sobrinha e à minha esposa Camila.

AGRADECIMENTOS

Ao Prof. Dr. José Butori Lopes de Faria, pela orientação e todo aprendizado durante estes anos de pesquisa no Laboratório de Fisiopatologia Renal e de Investigação de Complicações do Diabetes.

À Profª. Dra. Jacqueline Mendonça Lopes de Faria, pelas sugestões durante diferentes estágios deste trabalho.

Ao Prof. Dr. Rodrigo Bueno de Oliveira, pelas boas conversas e pelo apoio nos "quilômetros finais" dessa caminhada.

Aos colegas do LEMON e do CPC, pelos encontros em prol das pesquisas relacionadas ao *AGE reader* e pelos convites para participar das confraternizações.

Aos colegas do Laboratório de Fisiopatologia Renal e de Investigação deComplicações do Diabetes (antiga e nova geração), pela colaboração e auxílio nos experimentos e, em especial, ao meu amigo Antônio Calixto ("Antonito") que de forma prestativa e carinhosa sempre esteve ao meu lado, seja trabalhando ou dando boas risadas.

À equipe de profissionais da FCM / HC – UNICAMP: Departamentos de Nefrologia, Cardiologia (Prof. Dr. Wilson Nadruz), Anatomia Patológica, Laboratório de Análises Clínicas, funcionários do audiovisual, Comissão de Pesquisa – Estatística e Secretaria da Pós-Graduação por todo o suporte.

À FAPESP e ao CNPq, pelo apoio financeiro.

À Cláudia Barêa, pelo suporte terapêutico em momentos cruciais deste caminho.

Aos meus pais Adão e Anete por terem se dedicado à minha criação e desenvolvimento com amor, exemplo e compreensão, contribuindo para minha aprovação no vestibular da Faculdade de Medicina da Universidade Federal de Minas Gerais (UFMG), onde tudo começou.

À minha irmã Karina e à minha sobrinha Maria Clara, que apesar das adversidades da vida continuam lutando.

À minha esposa Camila, que esteve comigo desde o primeiro momento desta jornada. Por ser uma grande companheira – até na limpeza das gaiolas metabólicas! - e pelo seu constante amor, incentivo, apoio, paciência e compreensão.

“Mas é preciso ter manha
É preciso ter graça
É preciso ter sonho sempre
Quem traz na pele essa marca
Possui a estranha mania
De ter fé na vida”

(Fernando Brant e Milton Nascimento)

RESUMO

O estresse oxidativo é um dos mecanismos subjacentes mais importantes na patogênese da proteinúria em pacientes diabéticos. A epigalocatequina galato é uma substância que se mostrou efetiva na redução do estresse oxidativo e da doença renal em estudos pré-clínicos. Além disso, a redução na atividade da AMPK foi proposta como o ponto crítico na fibrose renal em modelos experimentais de DRC. A metformina foi capaz de melhorar a doença renal e reduzir a fibrose em estudos pré-clínicos de DRC, quando administrada antes ou no momento do insulto renal. Na presente série de estudos, propusemos investigar o efeito do polifenon E (epigalocatequina galato) na albuminúria de pacientes diabéticos com nefropatia e possível mecanismo envolvido. Investigamos, ainda, se a metformina é capaz de reduzir a progressão da DRC já estabelecida em ratos submetidos à nefrectomia subtotal 5/6 (Nx) e se tal efeito está associado à ativação da AMPK. Os estudos foram descritos nos seguintes artigos publicados:

Artigo 1: Os pacientes foram randomizados para 2 grupos: polifenon E (contendo 800 mg de epigalocatequina galato) e placebo. Após 12 semanas de tratamento, o polifenon E reduziu a albuminúria em 41%, enquanto no grupo placebo foi observado aumento de 2% ($p = 0,019$). A apoptose dos podócitos ($p = 0,001$) e a permeabilidade da albumina *in vitro* ($p <0,001$) foram maiores em podócitos humanos imortalizados expostos ao plasma de indivíduos diabéticos em comparação aos podócitos tratados com plasma de indivíduos normais. Neste artigo, concluímos que a administração de polifenon E reduz a albuminúria em pacientes diabéticos com dose máxima recomendada de inibidores do sistema renina- angiotensina (SRA). A ativação da via Wnt e a consequente redução na apoptose dos podócitos pode ter contribuído para tal efeito.

Artigo 2: Ratos machos Munich-Wistar adultos foram submetidos às cirurgias de nefrectomia subtotal 5/6 (Nx) ou sham. Trinta dias após a cirurgia, os ratos Nx que apresentavam pressão arterial sistólica > 170 mmHg e níveis de albuminúria >40mg/24h foram randomizados para 3 grupos: Sham, Nx e Nx + metformin (300mg/Kg/dia). Após 60 dias de tratamento, não observamos diferenças nos parâmetros da doença renal entre os ratos tratados com metformina e os não tratados. No entanto, após 120 dias, os ratos Nx tratados com metformina apresentaram

reduções significativas nos níveis de albuminúria e em marcadores de fibrose renal. Esses efeitos foram independentes de quaisquer outros efeitos sobre a pressão arterial ou a glicemia. O tratamento com metformina também foi capaz de ativar a AMPK renal e, portanto, melhorar a biogênese mitocondrial. Concluímos que a metformina pode interromper a progressão da doença renal estabelecida no modelo Nx, provavelmente, via ativação da AMPK.

Em resumo, a presente tese fornece evidências de que o Polifenon E, associado aos inibidores do SRA, é capaz de reduzir a albuminúria de pacientes com nefropatia diabética e de que tal efeito pode ser devido à ativação da via Wnt, com consequente redução na apoptose de podócitos. Adicionalmente, a metformina reduz a fibrose renal em ratos Nx e doença renal estabelecida, possivelmente pela ativação da AMPK.

ABSTRACT

Oxidative stress is one of the most important underlying mechanisms in the pathogenesis of proteinuria in diabetic patients. The epigallocatechin gallate is a substance that has been shown to be effective in reducing oxidative stress and kidney disease in preclinical studies. In addition, the reduction in AMPK activity has been proposed as a mechanism involved in renal fibrosis in experimental models of chronic kidney disease (CKD). Metformin was able to improve kidney disease and reduce fibrosis in preclinical studies of CKD, when administered before or at the time of renal insult. In the present series of studies, we proposed to investigate the effect of polyphenon E (epigallocatechin gallate) on albuminuria in diabetic patients with nephropathy and to investigate the possible mechanism involved. We furthermore investigated whether metformin is able to reduce the progression of CKD already established in rats submitted to 5/6 subtotal nephrectomy (Nx) and whether this effect is associated with AMPK activation. The studies were described in the following published articles:

Article 1: Patients were randomized to two groups: polyphenon E (containing 800 mg of epigallocatechin gallate) or placebo. After 12 weeks of treatment, polyphenon E reduced albuminuria by 41%, while in the placebo group an increase of 2% was observed (p = 0,019). Apoptosis of podocytes (p = 0,001) and permeability to albumin in vitro (p <0,001) were greater in immortalized human podocytes exposed to the plasma of diabetic individuals compared to podocytes treated with plasma from normal individuals. In this article, we concluded that the administration of polyphenon E reduces albuminuria in diabetic patients who receive the maximum recommended dose of renin-angiotensin system inhibitors. The activation of the Wnt pathway and the consequent reduction in apoptosis of podocytes may have contributed to this effect.

Article 2: Adult male Munich-Wistar rats underwent 5/6 subtotal nephrectomy (Nx) or sham surgery. Thirty days after surgery, Nx rats with systolic blood pressure> 170 mmHg and albuminuria levels> 40 mg / 24 h were randomized into three groups: Sham, Nx and Nx + metformin (300mg/Kg/day). After 60 days of treatment, we found no differences in renal disease parameters between rats treated with metformin and untreated rats. However, after 120 days, Nx rats treated with metformin showed significant reductions in albuminuria levels and in renal fibrosis markers. These effects were independent of any other effects on blood pressure or blood glucose. In addition,

metformin treatment was also able to activate renal AMPK and therefore improve mitochondrial biogenesis. We conclude that metformin can halt the progression of kidney disease established in the Nx model, probably via AMPK activation.

In summary, the present thesis provides evidence that Polyphenon E associated with RAS inhibitors is able to reduce albuminuria in patients with diabetic nephropathy and this effect may be secondary to the activation of the Wnt pathway with a consequent reduction in podocyte apoptosis. Additionally, metformin reduces renal fibrosis in Nx rats and established kidney disease, possibly by activating AMPK.

LISTA DE ABREVIATURAS E SIGLAS

ACC – acetyl –CoA carboxylase

ACEi – angiotensin-converting enzyme inhibitors

AMPK – AMP-activated protein kinase

ARB – angiotensin receptor blockers

BRA – bloqueadores do receptor de angiotensina

CKD – chronic kidney disease

DKK-1 – dickkopf-1

DM – diabetes melito

DRC – doença renal crônica

DN – diabetic nephropathy

EGCG – epigalocatequina gallato

eGFR – estimate glomerular filtration rate

GTP – green tea polyphenols

iECA – inibidores da enzima conversora de angiotensina

LRP6 – low-density protein 6

ND – nefropatia diabética

Nx – nefrectomia subtotal 5/6

OXPHOS proteins – complexes I-V of mitocondrial respiratory chain

pAMPK – phospho AMPK

pACC – phospho ACC

PGC1α – peroxisome proliferator- activated receptor-γ coactivator-1α

RAS – renin-angiotensin system

RFGe – ritmo de filtração glomerular estimado

SRA – sistema renina-angiotensina

TGF-β – transforming growth factor beta

TNF-α – tumor necrosis fator alpha

UACR – urinary albumin to creatinine ratio

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1. INTRODUÇÃO

1.1. Definição e classificação da doença renal crônica

A doença renal crônica (DRC) se caracteriza pela perda lenta, progressiva e irreversível da função renal. A DRC é definida pela diminuição do ritmo de filtração glomerular (RFG) ou pela presença de lesão renal (na Figura 1 é representada pela razão albumina creatinina na urina (UACR) sigla em inglês que corresponde à *urinary albumin creatinine ratio*) por três ou mais meses, independentemente da causa (KDIGO, 2012; Inker et al., 2014). A persistência do dano ou diminuição da função por pelo menos três meses é necessária para distinguir pacientes com DRC daqueles com lesão renal aguda (Levey et al., 2015). O RFG é geralmente considerado o melhor indicador da função renal geral e, quando está em declínio, é a marca registrada da doença renal progressiva (KDIGO, 2012; Chen et al., 2019).

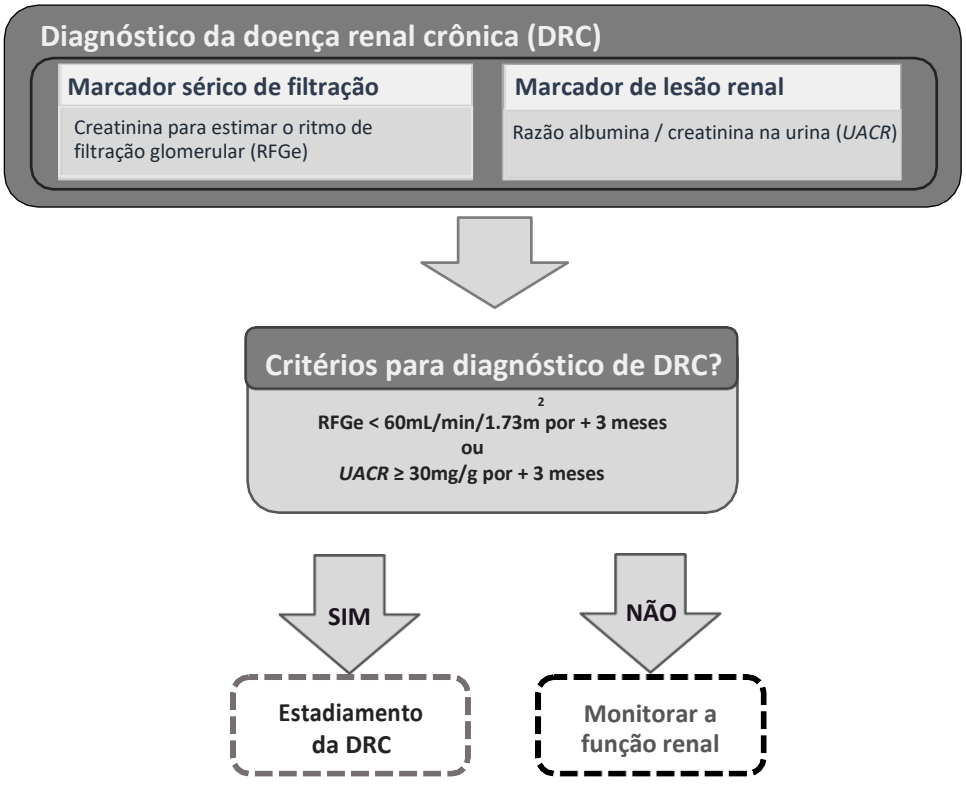

Figura 1. Diagnóstico da doença renal crônica (DRC). **Fonte: Adaptado de Chen et al., 2019**

Uma vez realizado o diagnóstico da DRC, o próximo passo é determinar o estadiamento, que se baseia no RFG e na albuminúria. O estadiamento da DRC é classificado em estágios (Figura 2) (KDIGO, 2012). Embora o RFG possa ser medido diretamente (Brown et al., 1991), o desenvolvimento de equações para estimá-lo (por exemplo, as equações da Colaboração em Epidemiologia da Doença Renal Crônica [CKD-EPI] e Modificação da Dieta no Estudo da Doença Renal [MDRD]) tem substituído amplamente a necessidade de mensuração direta na prática clínica. (Nailk et al., 2014; Grams et al., 2016).

A equação mais utilizada para estimar o RFG é a da equação do CKD-EPI 2009, sendo mais precisa do que a equação da MDRD, principalmente em pacientes com valores de RFG estimado (RFGe) maiores que 60 mL / min / 1,73 m2. (Levey et al. 2009). A creatinina sérica é o marcador utilizado com maior frequência para estimar a filtração glomerular (Levey et al. 2015).

			Categorias da albuminúria persistente		
			A1	A2	A3
			Normal a levemente aumentado	Moderadamente aumentado	Gravemente aumentado
			< 30 mg/g	30 – 300 mg/g	> 300 mg
Categorias da RFG (mL/min/1.73m^2)	E1	Normal ou aumentado	≥ 90		
	E2	Levemente reduzido	60 - 89		
	E3a	Leve / moderadamente reduzido	45 - 59		
	E3b	Moderado / gravemente reduzido	30 - 44		
	E4	Gravemente reduzido	15 - 29		
	E5	Falência renal	< 15		

Figura 2. Estadiamento da doença renal crônica pelo RFG e albuminúria, **Fonte: KDIGO, 2012.**

RFG indica ritmo de filtração glomerular, E indica estágio e A indica albuminúria; as categorias são agrupadas por risco de progressão da DRC, definido por declínio no RFG e aumento na albuminúria. Categoria RFG (acompanhada de uma redução de 25% no RFG estimada da linha de base) ou declínio sustentado no RFG estimado superior a 5 mL / min / 1,73m2 por ano. **Verde:** indica normal à baixo risco (se nenhum outro marcador doença renal e sem DRC); **amarelo:** baixo à moderado risco; **laranja:** moderado à alto risco; **vermelho:** risco alto (KDIGO, 2012; Chen et al., 2019).

A causa da DRC é geralmente classificada pela presença ou ausência de doença sistêmica e pela localização de anormalidade anatômica. Exemplos de doenças sistêmicas incluem diabetes melito (DM), doenças autoimunes, neoplasias e distúrbios genéticos nos quais o rim não é o único órgão afetado. As localizações anatômicas são divididas em doenças glomerulares, túbulo-intersticiais, vasculares e císticas/congênitas (KDIGO, 2012). O DM é a principal causa de doença renal crônica (KDIGO, 2012). Em 2016, 1 em cada 11 adultos, em todo o mundo, tinha DM; destes, mais de 80% viviam em países de baixa e média renda (WHO - World Health Organization accessed April 22, 2020; Chan et al. 2016), onde os recursos para o atendimento ideal são limitados. Estima-se que a hipertensão arterial sistêmica (HAS) afete um bilhão de indivíduos em todo o mundo (KDIGO, 2012; WHO accessed April 22, 2020) e é a segunda principal causa atribuída de DRC. A determinação da causa da DRC pode ter implicações importantes na prevenção, no tratamento e no prognóstico. Por exemplo, se a HAS e o DM forem diagnosticados precocemente, o controle adequado dos níveis pressóricos e glicêmicos podem prevenir o surgimento da DRC. (Chen et. Al, 2019).

1.2. Epidemiologia

De acordo com *Global Burden Disease*, o ônus da DRC medido em incidência, prevalência e morte por DRC aumentou substancialmente no mundo inteiro nos últimos 30 anos. Globalmente, em 2016, haviam mais de 21 milhões de casos incidentes de DRC, 276 milhões de casos prevalentes e quase 1,2 milhão pessoas morreram por causa da DRC (GBD Chronic Kidney Disease Collaboration, 2017; Xie et al., 2018). A prevalência de DRC foi estimada em 9% na população mundial, com os estágios 1 a 2 da DRC representando 5%; o estágio 3, 4%; estágio 4, 0,2%; estágio 5, 0,1%; diálise, 0,41% e transplante renal, 0,01%. Só no Brasil, em 2017, foi estimada uma prevalência de cerca de 17 milhões de pacientes em estágios diferentes da DRC (GBD Chronic Kidney Disease Collaboration, 2017). No mesmo ano, 1,2 milhão de mortes foram atribuídas ou estavam associadas ao diagnóstico de DRC - taxa de mortalidade que foi projetada para aumentar entre 2,2 milhões (em um cenário melhor) e até 4 milhões, no pior cenário, até 2040 (Foreman, 2018). Outras 1,4 milhão de

mortes por doenças cardiovasculares foram atribuídas à função renal comprometida. O *Global Burden Disease* classifica a DRC como a 12ª principal causa de morte entre 133 outras doenças (GBD Chronic Kidney Disease Collaboration, 2017). Ainda em 2016, a DRC resultou em mais mortes do que a tuberculose ou o HIV, e o número de mortes por DRC foi quase igual ao número de mortes devido a acidentes nas estradas (GBD Chronic Kidney Disease Collaboration, 2017). No Brasil, 35.550 pessoas perderam a vida devido à DRC em 2017 (GBD Chronic Kidney Disease Collaboration, 2017). No geral, observou-se uma variação significativa nas tendências demográficas e epidemiológicas entre as regiões geográficas e o ônus da DRC é mais evidente nas economias menos desenvolvidas, com desempenho do sistema de saúde abaixo do ideal (Xie et al., 2018, Chen et al., 2019).

O custo econômico com o tratamento dos pacientes com DRC é bastante elevado em muitos países desenvolvidos e em desenvolvimento. Nos Estados Unidos, o tratamento da DRC custa cerca de 48 bilhões de dólares por ano e o programa DRC terminal consome 6,7% do orçamento total do *Medicare,* para cuidar de menos de 1% da população coberta por planos de saúde. Um estudo realizado na Itália concluiu que os custos anuais para pacientes em tratamento com DRC antes da diálise foram estimados em 11 mil euros, contra 54 mil euros para pacientes em diálise (Xie et al., 2018). Na China estima-se que a economia perderá 558 bilhões de dólares na próxima década devido aos efeitos sobre a morte e a incapacidade atribuíveis a doenças renais e cardiovasculares crônicas. No Brasil, só no ano de 2018, foram gastos quase 3 bilhões de reais no tratamento renal substitutivo de pacientes com DRC terminal (WHO - World Health Organization accessed April 22, 2020).

1.3. Tratamento atual da DRC

1.3.1. Educação alimentar

O manejo dietético para impedir a progressão da DRC é controverso, pois grandes estudos mostraram resultados não conclusivos (Menon et al., 2009; Goraya et al., 2012; Chen et al., 2019). Entretanto, as diretrizes do *Kidney Disease Improving Global Outcomes* (*KDIGO*) recomendam que a ingestão de proteínas seja reduzida para menos de 0,8g/kg por dia, em adultos com DRC estágios E4-E5, e para menos de 1,3g/kg por dia em indivíduos nos demais estágios da DRC e em risco de progressão (KDIGO, 2012). Dietas com baixo teor de sódio (geralmente < 2g por dia) são recomendadas para pacientes com HAS, proteinúria ou sobrecarga de volume (KDIGO, 2012).

1.3.2. Controle adequado da pressão arterial

Muitas diretrizes fornecem algoritmos detalhados sobre quais agentes anti-hipertensivos devem ser utilizados para tratar a HAS em pessoas com DRC (Whelton et al., 2018). A presença e gravidade da albuminúria devem ser avaliadas para definir o melhor tratamento da HAS na DRC. O bloqueio do sistema renina-angiotensina-aldosterona, com um inibidor da enzima conversora de angiotensina (iECA) ou um bloqueador de receptores da angiotensina II (BRA), é recomendado para pacientes com DM e UACR (razão albumina creatinina na urina) de pelo menos 30 mg por 24 horas, ou para qualquer paciente com uma UACR de pelo menos 300 mg por 24 horas (KDIGO, 2012; Whelton et al., 2018). A terapia dupla com iECA e BRA não deve ser utilizada em pacientes com DRC, pois pacientes tratados com esse esquema apresentaram um desfecho pior (Whelton et al., 2018; Bandak et al., 2017). Os antagonistas dos receptores de aldosterona também podem ser considerados em pacientes com albuminúria, hipertensão resistente ou insuficiência cardíaca com fração de ejeção reduzida (Whelton et al., 2018, Ando et al., 2014; Bakris et al., 2015).

1.3.3. Controle glicêmico

O controle adequado do DM é muito importante nos pacientes renais crônicos, uma vez que pode retardar a progressão da DRC. A maioria das diretrizes recomendam valores de hemoglobina glicada próximo de 7,0% (UK Prosprective Diabetes Study Group, 1998; KDIGO, 2012; Bilo et al., 2015). Ajustes na dose diária dos agentes hipoglicemiantes orais podem ser necessários. Em geral, os medicamentos amplamente eliminados pelos rins (como, por exemplo, a glibenclamida) devem ser evitados, enquanto medicamentos metabolizados pelo fígado - e/ou parcialmente excretados pelos rins, tais como alguns inibidores da dipeptidil peptidase 4 [DPP-4] - podem exigir redução da dose, principalmente quando a TFG estimada ou mensurada cai abaixo de 30mL/min/1,73m2 (Bilo et al., 2015).

A metformina não deve ser usada em pacientes com menos de 30ml/min de TFG, pelo aumento no risco de acidose lática. As glifozinas são, provavelmente, o maior avanço no tratamento da ND desde os bloqueadores do sistema renina-angiotensina. As mesmas são agentes hipoglicemiantes potentes, capazes de reduzir a progressão da DRC associada ao DM tipo 2 e o risco de doenças cardiovasculares (Zinman et al., 2015; Perkovic et al., 2019). E assim como a metformina as glifozinas não são recomendadas em pacientes com TFG estimada ou mensurada abaixo de 30mL/min/1,73m2 (Neal et al., 2017; Perkovic et al., 2019).

1.3.4. Redução do risco de doenças cardiovasculares

A prevalência de doença cardiovascular é marcadamente maior entre indivíduos com DRC em comparação com aqueles sem DRC, portanto, um componente importante no manejo da DRC é a redução do risco cardiovascular. Recomenda-se que pacientes com 50 anos de idade ou mais, com DRC sejam tratados com estatina em dose baixa, independente do nível de colesterol de lipoproteína de baixa densidade (KDIGO, 2013; Tonelli et al., 2013). As diretrizes do JNC8 e do KDIGO recomendam pressões arteriais sistólicas e diastólicas < 140mmHg e < 90mmHg, respectivamente, entre adultos com DRC (baixo grau de evidência) (KDIGO, 2012; James et al., 2014). As diretrizes do KDIGO

recomendam ainda que adultos com UACR de pelo menos 30 mg por 24 horas (ou equivalente) tenham pressão arterial sistólica e diastólica mantidas abaixo de 130 x 80 mmHg (KDIGO, 2012).

1.3.5. Evitar drogas nefrotóxicas

Todos os pacientes com DRC devem ser aconselhados a evitar drogas nefrotóxicas. A administração rotineira de anti-inflamatórios não esteroidais (AINEs) na DRC não é recomendada, especialmente entre indivíduos que façam terapia com iECA ou BRA (KDIGO, 2012). Preparações intestinais à base de fosfato (formulações orais ou enema), disponíveis em farmácias e vendidos sem receita, podem levar a nefropatia aguda por fosfato (Rocuts et al., 2009). Os inibidores da bomba de prótons, muitas vezes prescritos sem indicação criteriosa, têm sido associados à incidência de DRC e relatos de casos de nefrite intersticial aguda (Lazaru et al., 2016).

1.3.6. Ajuste na dose de medicamentos

Ajustes na dosagem de medicamentos são frequentemente necessários em pacientes com DRC. A lista de medicamentos mais comuns que requerem redução de dose inclui a maioria dos antibióticos, os anticoagulantes orais, a gabapentina, a pregabalina, os hipoglicemiantes orais, a insulina, os quimioterápicos e os opiáceos (KDIGO, 2012). Os meios de contraste à base de iodo são contraindicados em indivíduos com lesão renal aguda e devem ser utilizados com cautela em pacientes com TFG estimada < 30mL/min/1,73m2, já que podem causar nefropatia induzida por contraste (Isaka et al., 2020).

Apesar do tratamento multifatorial supracitado por meio do controle glicêmico e da pressão arterial, incluindo doses máximas iECA ou BRA, bem como a administração de medicamentos hipolipemiantes, ajuste de doses de medicamentos e suspensão de drogas nefrotóxicas ser uma abordagem eficiente, não tem sido suficiente, já que muitos pacientes ainda evoluem para terapia renal substitutiva – diálise ou transplante renal (United States Renal Data System – USRDS, 2018; GBD Chronic Kidney Disease Collaboration, 2017). Assim, nos últimos dez anos, diversas pesquisas no campo da nefrologia - DRC

- têm se concentrado em identificar novos alvos terapêuticos, principalmente nas vias moleculares e celulares, tentando prevenir ou reverter a fibrose no tecido renal (Ruiz-Ortega et al., 2020) e evitar a progressão da DRC.

1.4. Por que testar a eficácia da epigalocatequina galato em pacientes com nefropatia diabética (DRC)?

O aumento progressivo da proteinúria em pacientes com diagnóstico de DM há mais de 15 anos (em média), seguido de perda gradativa da função renal - que resulta, na maioria dos casos, em doença renal crônica terminal - é a evolução clássica da nefropatia diabética (Alicic et al., 2017; Anders et al., 2018).

O tratamento atual da ND baseia-se nos controles glicêmico, lipídico e pressórico rigorosos, associados ao bloqueio do sistema renina-angiotensina (SRA) (Roscioni et al., 2013; Alicic et al., 2017). Para mais, a descoberta dos inibidores do cotransportador sódio-glicose 2 - SGLT2 (glifozinas) revolucionou o tratamento dos pacientes com diabetes tipo 2 (Pareek et al., 2016; Perkovic et al., 2019).

Novos estudos demonstram que os efeitos benéficos das glifozinas vão além do controle glicêmico. As glifozinas, ao inibirem a reabsorção renal da glicose, reduzem a pressão arterial sistêmica e induzem efeitos hemodinâmicos que levam a melhores resultados cardiovasculares e renais em pacientes com DM tipo 2 (DeFronzo et al., 2016). O controle glicêmico rigoroso melhora a hiperfiltração (estágio 1 da ND), a microalbuminúria (estágio 2 da ND) e a macroalbuminúria (estágio 3 da ND) (Choudhury et al., 2010). A insulina, as gliflozinas e alguns inibidores da DPP-4 são boas opções de controle do DM em pacientes com DRC. Entretanto, a hipoglicemia consequente à otimização dos hipoglicemiantes pode aumentar a mortalidade de pacientes com doença cardiovascular concomitante (Ismail-Beigi et al., 2010), impedindo um controle linear da glicemia e perpetuando a agressão inflamatória ao rim.

Diversas linhas de evidências sugerem que a hiperlipidemia possa ter um importante papel na progressão da doença renal crônica no DM, seja pelo efeito tóxico dos lípides nas células do mesângio ou pela promoção da aterosclerose intra-renal (KDIGO, 2012). Entretanto, o efeito do controle lipídico na instalação

e progressão da doença renal diabética permanece indeterminado. Estudos com fibrato e estatina em pacientes diabéticos demonstraram uma resposta parcial ao efeito benéfico desses medicamentos na função renal. No primeiro, houve uma elevação no “clearance” de creatinina, mas não ocorreu redução na albuminúria; já com as estatinas, ocorreu o contrário (Abe et al., 2011; Tonelli et al., 2013). O controle da pressão arterial é prioritário no manejo da ND: diminui a velocidade de perda da função renal nos diabéticos tipo 1 e reduz a incidência de microalbuminúria nos indivíduos com diabetes tipo 2 (Lopes de Faria et al, 2011; KDIGO, 2012). Os inibidores do SRA são os antihipertensivos considerados de primeira linha nesta população de pacientes, devido ao seu efeito renoprotetor, atuando nos fatores hemodinâmicos e metabólicos (Roscioni et al., 2013). A redução da progressão da doença renal no DM (através do uso de iECA) e bloqueadores do receptor de angiotensina (BRA), é independente do seu efeito na pressão arterial (KDIGO, 2012; Roscioni et al., 2013). No DM tipo 1, as duas classes de medicamentos reduziram a microalbuminúria e apenas o iECA diminuiu a progressão da nefropatia (Hsu F et al., 2017; Mauer et al.,2002).

Nos diabéticos tipo 2, as glifozinas - o maior avanço recente no tratamento da ND, juntamente com os inibidores do SRA, reduziram a proteinúria, a velocidade de perda da função renal e o risco cardiovascular associado (DeFronzo et al., 2016; Perkovic et al., 2019). O bloqueio agressivo do SRA – duplo bloqueio ou doses elevadas de um único inibidor - tem sido alvo de muitos estudos; porém, estas estratégias diminuem a proteinúria, sem melhorar o desfecho: nefropatia diabética clínica e, secundariamente, aumentam o risco de insuficiência renal aguda (Fried et al., 2013; Ahmed A et al., 2016). Portanto, essas últimas alternativas não devem ser utilizadas em pacientes com ND.

As modalidades terapêuticas mencionadas, embora bastante eficazes, são insuficientes no combate da ND, uma vez que o número de pacientes com DRC secudária ao DM continua crescendo (United States Renal Data System – USRDS, accessed october 10 2019).

A patogênese da ND é complexa e multifatorial. Neste processo, estão envolvidos fatores genéticos (Righetti AE et al., 2001; Freedman et al., 2007), alterações metabólicas e na hemodinâmica renal induzidas pela hiperglicemia (Cooper, 2001; Biwas et al., 2008), ativação intra-renal do sistema renina-

angiotensina-aldosterona (Yang T, et al., 2017), ativação de vias inflamatórias e estresse oxidativo (Lopes de Faria et al. 2011). Este último, tem sido considerado o mecanismo subjacente crucial no desenvolvimento das complicações microvasculares do diabetes.

Há mais de uma década, o Laboratório de Fisiopatologia Renal e de Investigação de Complicações do Diabetes da Faculdade de Ciências Médicas (FCM) da Universidade Estadual de Campinas (UNICAMP) vem produzindo uma série de evidências, em estudos pré-clínicos, da importância do estresse oxidativo nas lesões microvasculares do diabetes, incluindo a retinopatia (Silva KC et al., 2009; Rosales MA et al., 2010; Silva KC et al., 2010, Rosales MA et al., 2014) e a nefropatia (Biswas SK et al., 2008; Peixoto EB et al., 2009; Ribaldo PD et al., 2009; Lopes de Faria JB, 2011; Peixoto EB et al., 2012; Faria AM et al., 2012; Papadimitriou A et al., 2014, Papadimitriou A et al., 2015; Peixoto EB et al., 2015) diabéticas. Em relação à doença renal diabética, foi demonstrado em ratos, que intervenções que reduziam o estresse oxidativo - como o uso de tempol (um mimético da superóxido desmutase) (Peixoto et al., 2009; Peixoto et al., 2012), o chá verde (Riobaldo et al., 2009; Faria et al., 2012; Peixoto et al., 2015), o cacau (Papadimitriou et al., 2014) e a teobromina (Papadimitriou et al., 2015) - eram capazes não só de reduzir os parâmetros de estresse oxidativo, como também de melhorar os indicadores de doença renal como a albuminúria e o aumento renal de matriz extracelular (indicativo de fibrose renal). Especificamente em relação ao chá verde e o seu principal princípio ativo (a epigalocatequina galato) foi observado, inicialmente, que esse composto era capaz de melhorar a doença renal diabética experimental, por reduzir a geração de radicais livres induzida pela via NADPH oxidase – em particular inibindo a subunidade Nox4 (Ribaldo et al., 2009). Em seguida, foi demonstrado que o chá verde reduzia o desacoplamento da óxido-nítrico- sintase (condição que determina aumento na geração de superóxido) restabelecendo os níveis de tetrahidrobiopterina (BH4) - um cofator crucial na manutenção do equilíbrio entre óxido nítrico e radical superóxido (Faria et al., 2012). Finalmente, foi demonstrado que a apoptose de podócitos (células cruciais na manutenção da integridade da barreira de filtração glomerular e, portanto, na manutenção da albumina no interior do vaso sanguíneo), induzida pela alta glicose, era dependente da redução do receptor LRP6 da via Wnt, que determinava maior

interação entre a glicogênio sintase quinase 3 (GSK3) e o p53 (Peixoto et al., 2015). O emprego do chá verde ou da epigalocatequina galato era capaz de reverter essa sequência de eventos e, com isso, reduzir a apoptose dos podócitos; de forma que essa série de evidências pré-clínicas indicavam fortemente a pertinência de conduzir uma investigação a respeito da utilidade de ambos no tratamento da ND em humanos.

1.5. Por que investigar o efeito da metformina em modelo experimental de fibrose renal (DRC)?

A fibrose é definida pelo crescimento excessivo, endurecimento e/ou cicatrização de vários tecidos e é atribuída ao excesso de deposição de componentes da matriz extracelular, incluindo o colágeno (Wynn et al., 2008), se caracterizando pela perda da homeostase celular e a interrupção da arquitetura normal do tecido. Na maioria dos órgãos, essa resposta tecidual se desenvolve quando há lesão persistente ou recorrente no epitélio ou endotélio. O agente etiológico que induz tal lesão pode variar de agentes infecciosos a exposições tóxicas / metabólicas à autoimunidade e, em alguns casos, a causa da lesão nunca é identificada (Horowitz et al., 2019).

A resposta do hospedeiro à lesão tecidual é um processo complexo, que envolve um arranjo temporal e espacial preciso das células inflamatórias residentes e recrutadas (Thannickal et al., 2014). A primeira fase é caracterizada pelo início da cascata de coagulação e influxo de plaquetas, levando à formação de um coágulo de fibrina que também é rico em fibronectina. A agregação plaquetária auxilia na hemostasia e a degranulação plaquetária serve como uma rica fonte de citocinas e fatores de crescimento, incluindo TGF-β (Gurtner et al., 2008; Rockey et al., 2015). Segue-se, rapidamente, o recrutamento de neutrófilos e monócitos/macrófagos com amplificação da resposta inflamatória aguda, eferocitose de células mortas, fagocitose de tecido danificado e proteção contra invasão microbiana (Gurtner et al., 2008; Kisselva et al., 2008; Wynn et al., 2016).

A segunda fase do reparo é caracterizada pela proliferação e ativação de células efetoras. As células epiteliais proliferam e migram na matriz extracelular

(MEC) provisória para restabelecer a função de barreira. As células endoteliais proliferam e a angiogênese é evidente. Os fibroblastos derivados de diferentes populações progenitoras são recrutados. Em seguida, vem uma fase efetiva de deposição e remodelação da MEC, na qual predominam os fibroblastos acumulados. Essas células se diferenciam em "miofibroblastos", que são células contráteis do tipo músculo liso, caracterizadas pela presença de "fibras de estresse", compostas por alfa actina do músculo liso e filamentos de miosina. Tais miofibroblastos são os principais responsáveis pela deposição, remodelação, organização e maturação do tecido cicatricial (Bochaton-Piallat et al., 2016; Horowitz et al. 2019).

Com a conclusão do processo normal de reparo, os miofibroblastos são eliminados por apoptose e a arquitetura do tecido parenquimatoso é reconstruída (Desmoulière et al., 2003). A apoptose miofibroblástica anuncia a fase de resolução do reparo normal da ferida. Em contraste, a fibrose é caracterizada pela não degradação da MEC, devido à baixa sensibilidade dos miofibroblastos à apoptose, morte/eliminação reduzida de células senescentes, não interrupção das ligações cruzadas do colágeno depositado na MEC, proteólise e degradação reduzida de moléculas da matriz por captação celular e autofagia (Ricard-Blum et al., 2018; Horowitz et al., 2019).

Na DRC, a fibrose renal se inicia com a deposição de tecido fibroso no espaço entre túbulos e capilares peritubulares (matriz patológica). Tal matriz é rica em colágeno I e III, mas também contém constituintes da membrana basal (MB) do capilar normal, incluindo fibronectina e colágenos IV e V (Yoshioka et al., 1989; Nerlich et al., 1991). A fibrose intersticial interfere na função normal dos túbulos para mediar o transporte dos capilares e receber nutrientes da circulação. À medida que a DRC progride, a matriz fibrótica se expande e os néfrons e seus capilares de suporte são perdidos, resultando em diminuição do volume renal e comprometimento da perfusão (Duffield et al., 2014). As alterações fibróticas ocorrem também nos glomérulos (glomerulosclerose), que se constituem predominantemente de proteínas MB com quantidades menores de colágenos III e V. As lesões escleróticas no tufo glomerular (glomerulosclerose) consistem predominantemente de proteínas da membrana basal com quantidades menores de colágenos III e V, enquanto a fibrose

periglomerular e a fibrose nos crescentes glomerulares geralmente contêm níveis mais altos de colágeno I (Yoshioka et al., 1989; Nerlich et al., 1991; Stokes et al., 2001).

As arteríolas sofrem uma forma de fibrose chamada arteriolosclerose, resultando na perda da auto-regulação do fluxo sanguíneo e, finalmente, na redução da perfusão. O aparecimento de fibrose nesses compartimentos estruturais está frequentemente associado a angiogênese anormal e, posteriormente, à obliteração capilar (Duffield et al., 2014). No rim, assim como em outros órgãos, vários mediadores solúveis, incluindo fatores de crescimento e citocinas, além de alterações na matriz, foram implicados na patogênese da fibrose. Verificou-se que a maioria dos mediadores solúveis envolvidos na fibrogênese promovem a ativação dos miofibroblastos e a resistência à apoptose.

O TGF-β é o principal regulador da transição epitélio-mesenquimal e da MEC (Blobe et al., 2000; Hinz et al., 2015; Rockey et al., 2015). Para além de seu papel na diferenciação e ativação de miofibroblastos, o TGF-β diminui a suscetibilidade de fibroblastos à apoptose, pela ativação de vias de proteína-quinase pró-sobrevivência dos miofibroblastos (Rockey et al., 2015). Além dos miofibroblastos, o glomérulo e o interstício renal atraem um grande número de leucócitos. A maioria é da linhagem mielóide e incluem neutrófilos em contextos mais agudos, enquanto macrófagos e células dendríticas predominam em contextos crônicos. Em alguns casos de DRC, particularmente em doenças imunomediadas, os linfócitos T também estão presentes em grande número. Evidências inequívocas de estudos em humanos e camundongos indicam que os leucócitos ativados contribuem para o processo fibrogênico (Duffield et al., 2014). Macrófagos ativados podem danificar o tecido diretamente ou gerar citocinas profibróticas e outros fatores de crescimento, capazes de gerar certos constituintes da matriz *in vivo* (Lin et al., 2009).

Estimativas atuais sugerem que a doença fibrótica é responsável por quase metade de todas as mortes nos países desenvolvidos ocidentais (Wynn, et al., 2004). Apesar dos mecanismos que resolvem a fibrose estabelecida e restauram a função do tecido serem incompletos, estudos diversos em humanos e em modelos animais sugerem que o potencial de prevenir ou reverter

alterações fibróticas existe na maioria dos órgãos/tecidos estudados (Horowitz et al., 2018).

Recentemente, uma redução na atividade da proteína quinase ativada por AMP (AMPK) estabeleceu um importante elo causal entre doença metabólica e o desenvolvimento da fibrose em diferentes órgãos, incluindo fígado, pulmão e rim (Sharma et al., 2014; Nguyen et al., 2018; Rangarajan et al., 2018; Satriano et al., 2013). A AMPK é o principal sensor de energia da maioria das células (Declèves et al., 2011) e é ativada em resposta à depleção de ATP ou a uma proporção intracelular aumentada de AMP para ATP, que preserva a sobrevivência das células em condições de baixa energia (Hardie et al., 2012).

Consistente com o seu papel na manutenção da homeostase energética, durante um estresse celular energético, a AMPK ativa as vias catabólicas que geram ATP, enquanto desativa as vias biossintéticas que consomem ATP (Hardie et al., 2012). Estudos experimentais e clínicos identificaram uma estreita relação entre AMPK e fibrogênese hepática. A atividade da AMPK foi baixa em 28 pacientes com fibrose/cirrose avançada em comparação com pessoas saudáveis (Liang et al., 2018) e um estudo experimental mostrou que a inativação da AMPK inibe a expressão do transportador de glicose 2 (GLUT2) e suprime a captação celular de glicose, gerando um comprometimento no metabolismo da glicose e estimulando a replicação do vírus da hepatite C (HCV) nos hepatócitos, aumentando assim o grau de fibrose (Douglas et al., 2015).

Foi demonstrado em humanos com fibrose pulmonar idiopática (FPI) e em um modelo experimental de fibrose pulmonar em ratos, que a atividade da AMPK é menor em regiões fibróticas associadas a miofibroblastos metabolicamente ativos e resistentes à apoptose (Rangarajan et al., 2018). Em camundongos com DRC por obesidade, a redução na atividade da AMPK promoveu a inflamação renal precoce e a fibrose sustentada. Os autores demonstraram que a ativação da AMPK bloqueou completamente a vacuolização lipídica nas células dos túbulos proximais, devido a atividade reduzida da redutase da HMG-CoA (3-hidroxi-3-metilglutaril-coenzima A) e a fosforilação aumentada de ácidos graxos (AG) (Declèves et al., 2011).

A diminuição da fosforilação de AG tem sido proposta como uma das causas de deficiência de energia e fibrose renal. A fosforilação da acetil-CoA

carboxilase (ACC) é um dos principais controladores da fosforilação de AG. Em estados de privação de energia celular, a AMPK fosforila a ACC - que por sua vez aumenta a oxidação de FAO e, consequente, geração de ATP. Estudo recente demonstrou que a inibição da regulação da fosforilação da ACC pela AMPK em camundongos foi associada ao aumento da fibrose (Lee et al., 2018). Há evidências de que a ativação da AMPK reduziu acentuadamente o acúmulo glomerular de TGF-β, colágeno e fibronectina em modelos experimentais de nefropatia diabética (Mishra et al., 2008; Papadimitriou et al., 2014).

O mecanismo basal da ativação da AMPK na inibição do TGF-β ainda não é claro, entretanto, foi sugerido que, na DRC, uma redução na atividade da AMPK pode levar à fibrose renal através da ativação das vias do TGF-β - Smad2 (Papadimitriou et al., 2014) e Smad3 (Mishra et al., 2008). Uma outra via pela qual a ativação da AMPK protege as células em um estado de falta de energia envolve a estimulação de um importante regulador da biogênese mitocondrial: o co-ativador 1α do receptor ativado pelo proliferador de peroxissomo (PGC-1α). PGC-1α é um potente estimulador de muitas proteínas mitocondriais e sua ativação aumenta o conteúdo mitocondrial celular (Austin et al., 2012). Em estados de ativação reduzida da AMPK, também se espera que a atividade da PGC-1α seja reduzida (Jäger et al., 2007). Uma redução precoce e progressiva do conteúdo mitocondrial, potencialmente impulsionada pela atividade reduzida da AMPK e PGC-1α, parece estar ligada à inflamação renal precoce e às vias profibróticas (Lynch et al., 2017). Coincidentemente, a ativação da AMPK com drogas como ribonucleotídeo 5-aminoimidazol-4- carboxamida (AICAR) ou metformina reduziu a fibrose nos tecidos hepático, pulmonar e renal (Nguyen et al., 2018; Rangarajan et al., 2018; Satriano et al., 2013).

A metformina é o tratamento de primeira linha do DM tipo 2, devido à sua capacidade de reduzir a glicose no sangue em associação com efeitos benéficos nos lipídios plasmáticos, peso corporal e baixa incidência de eventos micro e macrovasculares (Corremans et al., 2019). É um dos antidiabéticos mais antigos e mais prescritos em todo o mundo.

Atualmente, é bem evidente que a metformina exerce efeitos pleiotrópicos além do seu efeito hipoglicemiante (De Broe et al., 2018). Em diversos estudos, a eficácia potencial da metformina na prevenção da fibrose foi demonstrada em

diferentes órgãos, incluindo pulmão (Rangarajan et al., 2018), fígado (Nguyen et al., 2018) e rim (Satriano et al., 2013). Estudo *in vivo* e *in vitro* demonstrou que a metformina melhorou a esteato-hepatite e a fibrose hepática (Nguyen et al., 2018). Já no pulmão, o tratamento com metformina foi capaz de reverter a fibrose bem estabelecida no tecido pulmonar (Rangarajan et al., 2018). Nos rins, a metformina também foi capaz de reduzir a fibrose renal em modelos experimentais de DRC pelo DM (Huiwen et al., 2019), dieta hiperlipídica (Tikoo et al., 2016), obstrução ureteral unilateral (Cavaglieri et al., 2015), cisplatina (Li et al., 2016) e nefrectomia subtotal 5/6 (Satriano et al., 2013). Nesses estudos, os efeitos benéficos de proteção renal da metformina parecem ter sido mediados pela ativação do AMPK. Uma limitação importante em todos eles é que a metformina foi iniciada antes ou no mesmo momento do início da lesão renal, na tentativa de prevenir a fibrose renal. A instituição do tratamento da fibrose renal antes ou concomitante a agressão renal inicial não ocorre na prática clínica.

2. HIPÓTESES E OBJETIVOS

O estresse oxidativo é um dos mecanismos subjacentes mais importantes na patogênese da proteinúria em pacientes diabéticos. A epigalocatequina galato é uma substância que se mostrou efetiva na redução do estresse oxidativo associado à ND em estudos pré-clínicos. É possível que o emprego da epigalocatequina galato possa ser útil no tratamento de pacientes com nefropatia diabética, reduzindo a albuminúria por diminuir a apoptose de podócitos.

Além disso, a redução na atividade da AMPK foi proposta como o ponto crítico na fibrose renal em modelos experimentais de DRC (Sharma et al., 2014). A metformina – droga hipoglicemiante ativadora da AMPK - foi capaz de melhorar a doença renal e reduzir a fibrose em estudos pré-clínicos de DRC, quando administrada antes ou no momento do insulto renal (Huiwen et al., 2019; Tikoo et al., 2016; Cavaglieri et al., 2015; Li et al., 2016; Satriano et al., 2013). É esperado que a metformina seja eficaz em reduzir a progressão da doença renal crônica já estabelecida, por ativar a AMPK e consequentemente reduzir a fibrose renal.

Objetivos específicos

- Investigar se o tratamento com polifenon E (epigalocatequina galato) é capaz de reduzir a albuminúria de pacientes com nefropatia diabética.
- Investigar o mecanismo de ação do polifenon E na redução da albuminúria. Testar se ocorre pela ativação da via Wnt e, consequente, redução da apoptose de podócitos.
- Investigar se o tratamento com metformina é capaz de reduzir a fibrose renal de ratos submetidos à nefrectomia subtotal 5/6 e doença renal estabelecida (proteinúria e hipertensão arterial sistêmica) e se tal efeito está associado ao aumento da atividade da AMPK.

3. RESULTADOS

Artigo 1: *The use of green tea polyphenols for treating residual albuminuria in diabetic nephropathy: A double-blind randomised clinical trial*

SCIENTIFIC REPORTS

OPEN

The use of green tea polyphenols for treating residual albuminuriain diabetic nephropathy: A double-blind randomised clinical trial

Received: 28 January 2016
Accepted: 31 May 2016
Published: 20 June 2016

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Prior research has shown that in experimental diabetes mellitus, green tea reduces albuminuria by decreasing podocyte apoptosis through activation of the WNT pathway. We investigated the effect of green tea polyphenols (GTP) on residual albuminuria of diabetic subjects with nephropathy. We conducted a randomised, double-blind study in 42 diabetic subjects with a urinary albumin-creatinine ratio (UACR) >30 mg/g, despite administration of the maximum recommended dose of renin-angiotensin (RAS) inhibition. Patients were randomly assigned to two equal groups to receive either GTP (containing 800 mg of epigallocatechin gallate, 17 with type 2 diabetes and 4 with type 1 diabetes) or placebo (21 with type 2 diabetes) for 12 weeks. Treatment with GTP reduced UACR by 41%, while the placebo group saw a 2% increase in UACR ($p = 0.019$). Podocyte apoptosis ($p = 0.001$) and *in vitro* albumin permeability ($p < 0.001$) were higher in immortalized human podocytes exposed to plasma from diabetic subjects compared to podocytes treated with plasma from normal individuals. In conclusion, GTP administration reduces albuminuria in diabetic patients receiving the maximum recommended dose of RAS. Reduction in podocyte apoptosis by activation of the WNT pathway may have contributed to thiseffect.

Multifactorial treatment by means of blood glucose and blood pressure control, including maximum doses of angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), as well as administration of lipid lowering drugs, is an efficient, though insufficient, approach to treatment for diabetic nephropathy (DN)[1]. Despite the availability of so many effective interventions, DN remains the main cause of end-stage renal disease in most parts of the world[2].

In patients with DN, post hoc analyses of trial outcomes have demonstrated a robust relationship between the magnitude of albuminuria reduction and the slowing of chronic kidney disease (CKD) and reduced rates of cardiovascular events[3–8]. In addition, the high residual risk in diabetic nephropathy is directly related to the residual albuminuria in these patients[9]. Therefore, new treatment options must be added to the current armamentarium, especially drugs that can lower residual risk factors without increasing adverse events.

Preclinical studies in diabetic nephropathy[10–12] and in rapidly progressive glomerulonephritis[13] have demonstrated that green tea (GT) is able to reduce albuminuria and other markers of renal damage. In experimental DN studies, GT was able to reduce oxidative stress markers by inhibiting NOX4, as well as nitric oxidase synthase (NOS) uncoupling[10,11]. In addition, we recently demonstrated that under experimental diabetic conditions, GT reduces podocyte apoptosis by activating the WNT pathway[12]. Interestingly, GT was not able to reduce podocyte apoptosis when podocytes exposed to high glucose were treated with dickkopf 1 (DKK-1). DKK-1 is a blocker of low-density lipoprotein receptor-related protein 6 (LRP6), a receptor of the WNT pathway that is involved in podocyte death under conditions of high glucose exposure[14]. We concluded that GT could ameliorate albuminuria by reducing podocyte apoptosis through activation of the WNT pathway[12].

A recent meta-analysis of studies in human subjects concluded that moderate consumption of GT reduces the risk of cardiovascular events and stroke by enhancing endothelial-dependent vasodilation[15]. In addition, the use

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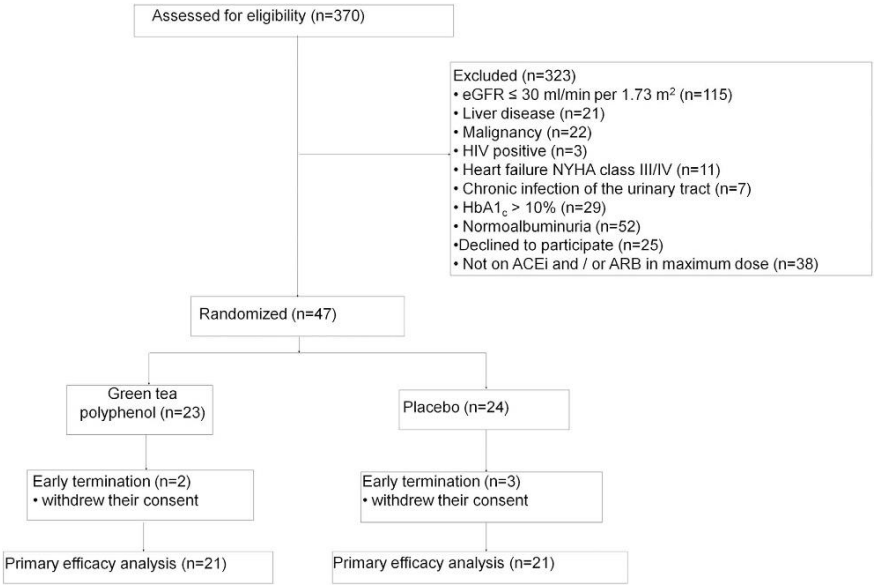

Figure 1. Flow diagram of the trial. eGFR, estimated glomerular filtration rate; HIV, human immunodeficiency virus; NYHA, New York Heart Association; HbA1c, glycated hemoglobin; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

of the main GT catechin, epigallocatechin gallate (EGCG), administered in doses of up to 800 mg daily, is safe for healthy postmenopausal women and reduces their LDL-cholesterol and plasma insulin levels[16]. To our knowledge, no study in humans has evaluated the potential efficacy of GT in treating patients with DN.

The aim of this study was to investigate whether green tea polyphenols (GTP) can reduce residual albuminuria in diabetic subjects receiving the maximum recommended dose of ACE inhibitors and/or ARBs and to test the hypothesis that GTP exerts its antiproteinuric effect via reduction of podocyte apoptosis by activating the WNT pathway.

Results

The screening phase of this trial started in November 2013; the treatment phase was completed by December 2014. As shown in the flow diagram (Fig. 1), 47 patients were enrolled in the study and randomly assigned to one of two groups to receive either GTP or placebo (Table 1). Two patients in the GTP group and three patients in the placebo group were ultimately excluded from final analysis of the study due to withdrawal of their consent and unavailability of final laboratory data. Eventually, 21 patients in each group completed the 12-week treatment phase of thetrial.

Primary endpoint. The same regime reported at baseline for RAS inhibition was maintained in all 42 patients who completed the study. Patients receiving GTP had a median (range) UACR of 210 mg/g (39–1267) at baseline, which was reduced to 133 mg/g (10–1812). Patients receiving placebo, on the other hand, experienced an increase in UACR, from 427 mg/g (77–4051) at baseline to 452 mg/g (43–4802) by week 12. After 12 weeks of treatment, GTP had reduced the geometric mean of % change from baseline (95% confidence interval [CI]) of UACRby 41%(−0.64/0.96), whilein theplacebo groupatinyincreaseof 2%(−0.13/0.45) wasnoticed ($p = 0.019$) (Fig. 2). The beneficial effect of GTP on albuminuria was maintained even when we included only patients with diabetes mellitus (DM) type 2 in the analyses, resulting in a 37% reduction vs. a 4% increase ($p = 0.03$) for the GTP and placebo groups, respectively. Notably, 19% of patients in the GTP group and none in the placebo group moved from macroalbuminuria or microalbuminuria to microalbuminuria or normoalbuminuria ($p = 0.03$).

Secondary endpoints. Median values for changes in 24-hour systolic and diastolic blood pressures, body mass index (BMI), glycated haemoglobin (HbA1c), estimated glomerular filtration rate (eGFR) values, and serum C-reactive protein (CRP) were not significantly different between the two groups (Table 2). However, the addition of GTP to the existing treatment regimen significantly reduced mean serum DKK-1 ($p = 0.004$) and tumor necrosis factor alpha (TNF-α; $p = 0.003$) with no significant change in urinary 8-isoprostane concentrations (Table 3).

Adverse events. No patients withdrew from the study because of adverse events associated with treatment. One patient developed diarrhea and another had dyspepsia after GTP treatment. One patient reported dizziness after placebo treatment.

***In vitro* studies.** *Plasma from diabetic patients increased albumin permeability and markers of apoptosis in immortalized human podocytes (iHPs).* Treatment of iHPs with plasma from diabetic subjects resulted in increased albumin permeability ($p < 0.001$, Fig. 3) and markers of apoptosis (caspase 3 activity, $p < 0.001$ and terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL], $p = 0.001$) (Fig. 4) as compared to

Characteristic	Green tea polyphenol ($N = 23$)	Placebo ($N = 24$)
Age–yr	63 (60–65)	59 (49–63)
Male sex–no. (%)	11 (47.8)	16 (66.7)
DM type 2–no. (%)	23 (100)	19 (79)
Known duration of DM–yr	16 (12–20)	19 (13–22)
Use of insulin–no. (%)	17 (73.9)	19 (79.2)
Use of statins–no. (%)	19 (82.6)	22 (91.7)
Use of aspirin–no. (%)	17 (73.9)	17 (70.8)
Body-mass index–kg/m^2	30.6 (27.5–34.7)	32.7 (28.6–35.5)
Waist circumference–cm	107 (99–113)	114 (99–119)
Diabetic retinopathy–no. (%)	19 (82.6)	17 (70.8)
ACEi/ARB/ACEi+ARB–no.	10/12/1	7/14/3
Systolic blood pressure–mmHg		
Office	151 (140–159)	140 (131–160)
24 hour	140 (128–144)	132 (118–139)
Diastolic blood pressure–mmHg		
Office	89 (81–97)	84 (76–99)
24 hour	76 (70–82)	73 (69–79)
Fasting plasma glucose–mg/dL	136 (118–180)	143 (99–194)
Glycated hemoglobin–%	7.7 (7.3–8.3)	8.2 (7.5–9.2)
Serum triglycerides–mg/dL	163 (110–195)	144 (97–207)
Serum total cholesterol–mg/dL	157 (136–169)	156 (147–186)
Serum LDL-C–mg/dL	84 (68–90)	86 (69–91)
Serum HDL-C–mg/dL	40 (33–46)	42 (36–51)
Serum CRP–mg/dL	0.40 (0.08–0.72)	0.23 (0.08–0.47)
eGFR–mL/min per 1.73 m$^{2\#}$	55.7 (44.3–71.7)	65.6 (46.2–85.6)
UACR–mg/g[&]	210 (39–1267)	427 (77–4051)
Micro/Macroalbuminuria–no. (%)[¶]	16 (70)/7 (30)	10 (42)/14 (58)

Table 1. Baseline characteristics of patients[^]. Abbreviations: DM, diabetes mellitus; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CRP, C-reactive protein; UACR, urinary albumin-to-creatinine ratio. [^]Values are median and interquartile ranges unless otherwise noted. [#]The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration formula. [&]Values are median (range). [¶]Microalbuminuria, UACR = 30–300 mg/g; macroalbuminuria, UACR >300 mg/g.

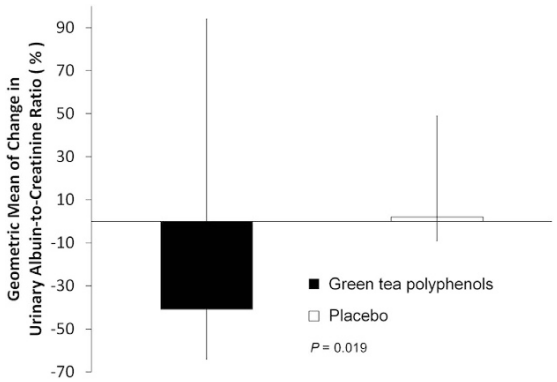

Figure 2. Patients in the green tea polyphenol group (n = 21) experienced a significant reduction in UACR, while patients in the placebo (n = 21) group experienced a small increase. Geometric mean of % change in urinary albumin-creatinine ratio from baseline to the end of the study. Vertical bars represent the 95% confidence intervals. *P* value is for comparison between the green tea polyphenol and the placebo groups.

podocytes treated with plasma from control subjects. Notably, we did not observe differences in these same parameters when we compared podocytes treated with plasma from control subjects with podocytes treated with plasma from diabetic patients who received GTP (Figs 3 and 4).

	GTP ($N = 21$)	Placebo ($N = 21$)	P value between groups
Δ 24-hour systolic BP–mmHg	−0.02 (−0.03/0.01)	−0.02 (−0.08/0.04)	0.62
Δ 24-hour diastolic BP–mmHg	−0.01 (−0.06/0.05)	0.01 (−0.02/0.08)	0.35
BMI–kg/m^2	0.01 (0.00/0.02)	0.00 (−0.01/0.01)	0.32
HbA1c–%	0.01 (−0.06/0.07)	0.02 (−0.04/0.05)	0.86
CRP–mg/dL	0.15 (−0.30/0.40)	0.00 (−0.28/0.60)	0.29
eGFR–mL/min per 1.73 m$^{2\#}$	−0.07 (−0.18/0.02)	−0.01 (−0.13/0.09)	0.42

Table 2. Change from baseline in the two groups. Data are expressed as median of percentage change between the two evaluation points, baseline and 12 weeks (interquartile range). #The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration formula. Abbreviations: GTP, green tea polyphenol; BP, blood pressure; BMI, body-mass index; HbA1c, glycated hemoglobin; CRP, C-reactive protein.

	GTP	Placebo	P Value between groups
DKK-1 (pg/mL)			
Median baseline (IQR)	787 (631/948)	839 (495/1063)	0.84
Δ Median after 12 weeks (IQR)	−0.39 (−0.62/−0.26)	0.01 (−0.05/0.13)	<0.001
TNF-α (pg/mL)			
Median baseline (IQR)	13.5 (11.3/17.5)	14.5 (11.8/15.1)	0.82
Δ Median after 12 weeks (IQR)	−0.16 (−0.28/−0.06)	0.06 (−0.03/0.15)	<0.001
8-isoprostane levels (fg/mg creatinine)			
Median baseline (IQR)	8.9 (7.1/2.5)	7.5 (4.5/15.6)	0.60
Δ Median after 12 weeks (IQR)	0.09 (−0.12/0.42)	0.05 (−0.06/0.33)	0.87

Table 3. Median and change from baseline of the two groups. Abbreviations: GTP, green tea polyphenol; DKK-1, Dickkopf WNT pathway inhibitor 1; TNF-α, tumor necrosis factor alpha.

Addition of DKK-1 to plasma from diabetic subjects who received GTP reversed the protective effect on albumin permeability and podocyte apoptosis markers. To test the possible contribution of WNT pathway activation to increased albumin permeability and podocyte apoptosis markers, we treated plasma from diabetic patients who received GTP with DKK-1, a WNT inhibitor. Interestingly, the protective effect of GTP was eliminated by the addition of DKK-1 (Figs 3 and 4).

Addition of EGCG to plasma of diabetic subjects reduced albumin permeability and markers of podocyte apoptosis. Addition of EGCG (436 nM) to plasma from diabetic subjects who did not receive GTP reduced albumin permeability ($p = 0.01$) and markers of podocyte apoptosis ($p = 0.007$) to the levels observed in podocytes treated with plasma from normal subjects (Figs 3 and 4).

Discussion

To our knowledge, this study is the first to demonstrate the efficacy and safety of adding GTP to maximum doses of ACE inhibitors and/or ARBs in order to reduce residual albuminuria in patients with diabetic nephropathy. Improvement in glomerular barrier selectivity due to reduction in podocyte apoptosis as a result of decreased plasma DKK-1 and WNT pathway activation may be the possible mechanism behind this observed efficacy. It is also possible that inhibition of inflammatory mediators (such as TNF-α) may have contributed to the beneficial effect of GTP[17]. The findings of the current study confirm in a clinical setting our preclinical study that suggested the ability of green tea to reduce albuminuria in diabetic nephropathy, probably by diminishing podocyte apoptosis through activation of the WNT pathway[12].

Previous studies have demonstrated that plasma DKK-1 is indeed elevated in type 2 diabetic patients in comparison to plasma from normal subjects[18,19]. In addition, elevated plasma concentrations of DKK-1 are associated with macrovascular disease in patients with type 2 DM[18], as well as endothelial dysfunction and platelet activation[19]. Interestingly, plasma concentrations of DKK-1 can be reduced with improved glycemic control and low-dose aspirin treatment[19]. In the present study, we demonstrated that GTP reduces plasma concentrations of DKK-1 independently of its effect on glycemic and blood pressure control.

DKK-1 is a major regulator of the WNT pathway, a group of highly conserved secreted mediators that regulate a wide range of cellular processes, such as proliferation and differentiation, survival, cell fate determination, and migration[20]. Scant but controversial evidence exists for the role of the WNT pathway in podocyte apoptosis. It has been suggested that in experimental DM, high glucose may activate the WNT pathway through stimulation of the transient receptor potential channel 6 (TRPC6), which eventually contributes to podocyte apoptosis[21]. On the contrary, Kato and colleagues have suggested that down-regulation of the WNT pathway in podocytes might in fact enhance apoptosis susceptibility[14]. This last suggestion was confirmed by our recent observations, wherein we demonstrated—both *in vivo* and *invitro*—that high glucose or blockage of the WNT receptor, LRP6, with DKK-1

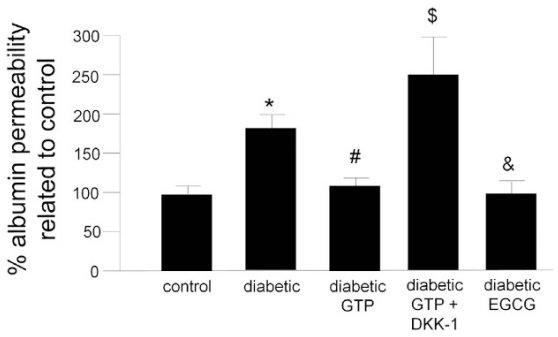

Figure 3. Analysis of the filtration barrier function of the podocyte monolayer by an albumin efflux assay. Differentiated iHPs were incubated with plasma from each healthy controls (n = 3) and plasma from each diabetic subjects before (n = 3) or after 12 weeks treatment with GTP (n = 3) and albumin influx through podocyte monolayer was assessed after 6 hours, as described in methods. Data are presented as mean and vertical bars represent the standard deviation. $^{*}P < 0.001$ vs. control, $^{\#}P = 0.003$ vs. diabetic, $^{\$}P < 0.001$ vs. diabetic GTP, $^{\&}P = 0.01$ vs. diabetic. GTP, green tea polyphenol; DKK-1, dickkopf 1, EGCG, epigallocatechin gallate.

or silencing RNA increases glycogen synthase kinase 3 beta (GSK3β), its interaction with p53, and ultimately podocyte apoptosis[12]. GSK3β is involved in WNT pathway regulation[22]. Its activation promotes proteasomal degradation of the transcription factor β-catenin and inhibits cytoplasmic accumulation and subsequent nuclear translocation[22]. Therefore, WNT target genes are not transcripted by β-catenin[22]. We are unaware of any study that has investigated the role of DKK-1/WNT pathway in podocyte apoptosis in human subjects.

Previous research has shown that in cultured podocytes, plasma from diabetic subjects with nephropathy induces apoptosis, as assessed by cleaved caspase 3[23]. Herein, we confirmed that plasma from diabetic patients with nephropathy promotes podocyte apoptosis, as assessed by cleaved caspase 3 and TUNEL assay (Fig. 4), likely due to an increased concentration of plasma DKK-1. This suggestion is supported by our observation that the protective effect of plasma from diabetic patients who received GTP was lost when we treated the podocytes with DKK-1 (Fig. 4). Similar results were also observed in an *in vitro* model of albumin permeability (Fig. 3).

We could not detect a significant correlation of UACR with DKK-1, nor with TNF-α. These findings may be explained by our small sample size, and hence the low power of this study to detect such correlations. However, when compared with placebo, GTP therapy significantly reduced DKK-1 and TNF.

The main limitations of our study and our interpretation of its results are the small sample size and the short duration of the treatment phase. Moreover, in addition to albuminuria, future studies should consider other outcome measures, such as time to renal replacement therapy.

In conclusion, to our knowledge this is the first randomised, double-blind, placebo-controlled clinical trial to demonstrate the possible efficacy and safety of GTP addition to RAS inhibitors in attenuating residual albuminuria in patients with diabetic nephropathy. This observed efficacy may be due to the capacity of GTP to reduce podocyte apoptosis as a result of reduction of DKK-1. Future multicenter randomized trials with larger samples sizes are necessary to confirm the long-term efficacy and safety of adding GTP to RAS inhibitors with the goal of minimizing the progression of diabetic nephropathy.

Materials and Methods

This randomised, double-blind, placebo-controlled, single-center phase 2 study was conducted in compliance with local and national regulations, Good Clinical Practices guidelines, and Declaration of Helsinki Principles and it was approved by the local Institutional Review Board (Comitê de Ética em Pesquisa, Faculdade de Ciências Médicas, UNICAMP, 18445613.3.0000.5404). All patients signed informed consent before enrollment in the study.

Patients. Eligible patients, identified during routine visits to the Diabetic Nephropathy Clinic at the University Hospital of the State University of Campinas (UNICAMP), were 18 years of age or older, had been previously diagnosed with DM type 1 or 2, and had persistent micro- or macroalbuminuria (UACR over 30 mg/g as determined by three consecutive urine samples obtained on different days) despite treatment with maximum doses of ACE inhibitors and/or ARBs for at least 8 weeks prior to the screening. Included patients also had HbA1c levels <10%, and regularly used insulin and/or oral glucose lowering agents. Exclusion criteria included autoimmune disease, human immunodeficiency virus (HIV) infection, viral hepatitis, neoplasia, pregnancy and lactation, eGFR below 30 mL/min per 1.73 m^2 as estimated by the CKD Epidemiology Collaboration (CKD-EPI) equation, chronic urinary tract infection, chronic heart failure of New York Heart Association (NYHA) class III or IV, recent history of coronary artery disease, cerebrovascular accidents, history of alcohol dependency or drug abuse, any psychiatric or neurological conditions preventing mindful consent to the study and/or adherence to the study protocol, and intolerance to green tea.

Randomization and Study Design. We screened 370 patients with a diagnosis of diabetes. Of these patients, 47 patients met the inclusion criteria and were randomly assigned to two groups: 24 patients were treated

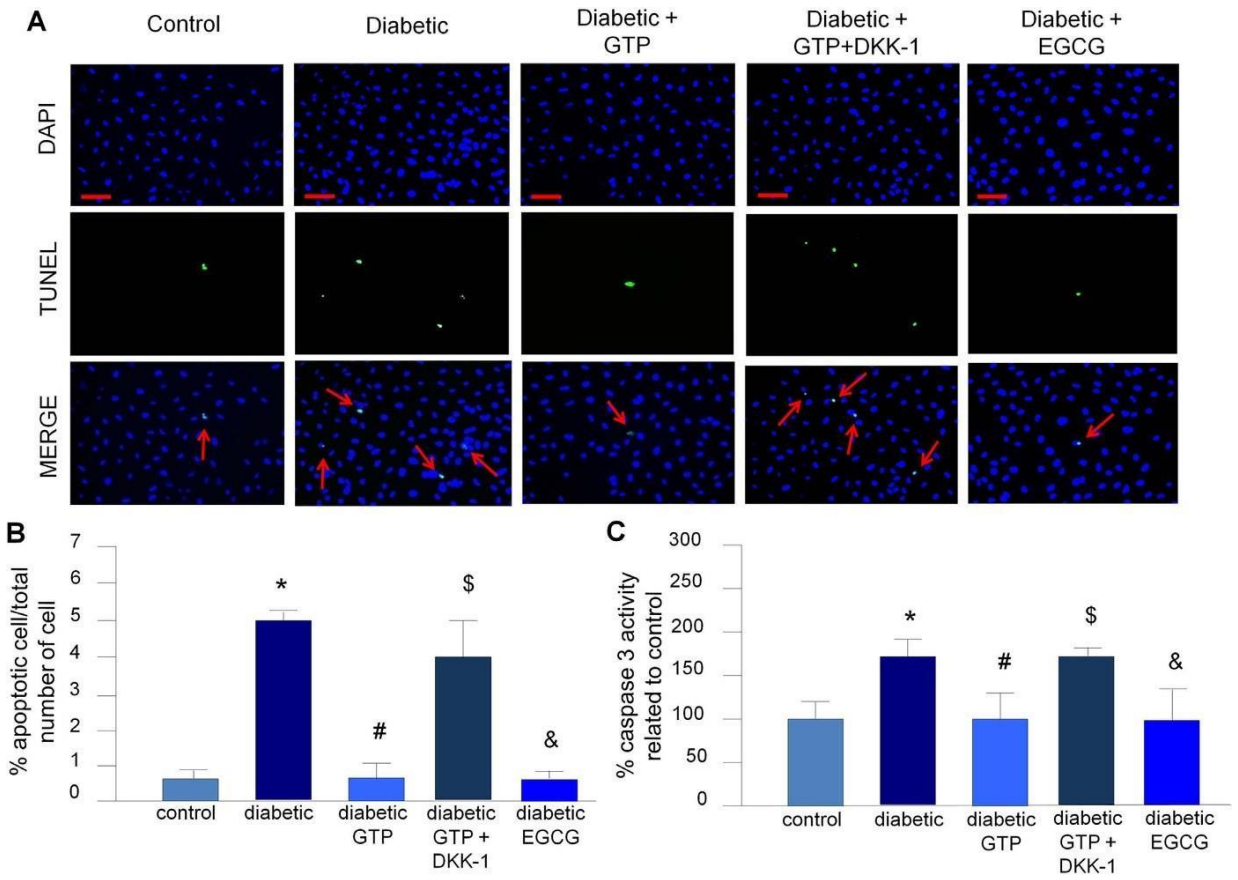

Figure 4. Analysis of podocyte apoptosis. (**A,B**) TUNEL assay, scale bars 50 μm. Bars in B represent the median of three human plasma samples derived from an average of ten fields. Vertical bars represent the standard deviation. $N = 3$ for each condition. $^{*}P = 0.01$ vs. control, $^{\#}P = 0.01$ vs. diabetic, $^{\$}P < 0.001$ vs. diabetic GTP, $^{\&}P < 0.001$ vs. diabetic. (**C**) Caspase-3 activity. Data are presented as mean ± SD; $N = 3$ for each condition. $^{*}P < 0.001$ vs. control, $^{\#}P < 0.001$ vs. diabetic, $^{\$}P < 0.001$ vs. diabetic GTP, $^{\&}P = 0.007$ vs. diabetic. GTP, green tea polyphenol; DKK-1, dickkopf 1, EGCG, epigallocatechin gallate.

with a maximum dose of ACE inhibitors and/or ARBs plus GTP, and 23 patients were treated with a maximum dose of ACE inhibitors and/or ARBs plus placebo. The patients received either four capsules of GTP (tea polyphenol, TP98, MedKoo Bioscience, Chapel Hill, NC 27516-6222, USA) per day, corresponding to 800 mg of EGCG, or placebo for 12 weeks. The percentage of EGCG, epigallocatechin, and epicatechin present in this GTP was confirmed by our laboratory (Supplementary Table 1). The dosage was chosen because it has been shown to be safe and because it might be effective for lowering LDL cholesterol[16]. All drugs and placebo tablets were similar in size, shape, weight, and color. The website Randomization.com (http://www.randomization.com) was used to generate the randomization list. All drug and placebo tablets were prepared by Dermage (Campinas, São Paulo, Brazil), and prepacked bottles were numbered for each patient according to the randomization sequence. All clinical investigators, laboratory personnel, and patients were masked to the treatment assignment. To avoid researcher influence, the randomization list was generated and maintained by trained personnel in a different location from thestudy.

Procedures and Outcomes. Patients were evaluated at baseline and then again after 12 weeks. At these two time points, patients provided three samples of first morning urine to determine albuminuria (primary endpoint) and a complete physical examination was performed, including office and 24-hour blood pressure, and BMI. A fasting blood sample was also obtained for determining secondary endpoints including: eGFR, glycemia, HbA1c, lipid profile, CRP, plasma concentration of DKK-1, TNFα, and urinary 8-isoprostane. Because of our recent preclinical observation[12] showing the potential of green tea to activate the WNT pathway and to reduce albuminuria under diabetic conditions, we post hoc estimated the plasma concentration of DKK-1, a WNT inhibitor, as a secondary outcome. We also post hoc estimated plasma TNF as a marker of inflammation. Patient adherence to the study was evaluated by monthly phone calls and by tablet counts at the end of the study. Adverse events were assessed during the visits and by phone calls.

Laboratory procedures. Urine samples taken over three consecutive mornings, were used to test for albuminuria at the beginning and the end of the study (Nephelometry, BN II System, Siemens, Germany). Ambulatory blood pressure was monitored over 24 hours (Spacelabs MAPA 90207, Washington, USA) and was measured

every 15 minutes during the day and every 30 minutes during the night. Hemoglobin concentration (Sysmex XE-2100, Sysmex Corp., Kobe, Japan), plasma potassium, sodium, creatinine, urea, and lipids (Roche/Hitachi MODULAR P, Roche Lab Systems, Germany) were measured by autoanalyser, CRP was measured by nephelometry (BN ProSpec System, Siemens), and HbA1c was determined using high-performance liquid chromatography (HPLC, Bio-Rad VARIANTII, USA). Plasma DKK1 (R&D, Minneapolis, MN, USA), TNFα (R&D), and urinary 8-isoprostane (ABCAM, Cambridge, MA, USA) were determined using commercially available ELISA kits in accordance with themanufacturer's instructions.

Human podocyte culture. Conditionally iHPs (M.A. Saleem, Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK) were provided by Luigi Gnudi (King's College, London, United Kingdom) and were derived and cultured as reported by Saleem *et al.*[24]. Differentiated podocytes cell passage numbers were between 12 and 17, with batches of cells from the same passage number used for each set of experiments. Replicate experiments were performed (the number of experiments is stated in each case below) and representative results are shown. After overnight serum starvation, differentiated cells were exposed to the treatments for the indicated duration. The concentrations of treatments used in all experiments were chosen after carrying out a thiazolyl blue tetrazolium bromide assay (Supplementary Fig. 1).

Human podocyte treatments. Starved iHPs were incubated with 4% plasma from each healthy control ($n = 3$) or from each type 2 diabetic patient treated with GTP, obtained before treatment ($n = 3$) or after 12 weeks of treatment with GTP ($n = 3$). Cells were incubated for 6 hours in the presence or absence of a low-density lipoprotein receptor-related protein (LRP) 6 (LRP6) blocker (100 ng/mL DKK-1) (R&D, Minneapolis, MN, USA) or EGCG (436 nM). This concentration of EGCG corresponds to the plasma concentrations obtained after administration of 800 mg of EGCG to healthy controls[25]. These experiments were performed at least twice. Data from the healthy controls and diabetic subjects used in these *in vitro* experiments are presented in Supplementary Table 2. Plasma from diabetic patients and control subjects was obtained at fasting state.

Caspase-3 activity assay. Caspase-3 activity in iHPs was analyzed using a colorimetric assay[26].

TUNEL assay. The TUNEL method was applied to iHPs using a DeadEnd Fluorometric TUNEL System detection kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The quantitative analysis of apoptosis in the cultured podocyte cells was carried out by counting the number of positive cells per total number of cells per square millimeter[12]. Ten microscopic images were evaluated for each individual.

Albumin influx assay. A simple albumin influx assay was used to evaluate the filtration barrier function of the podocyte monolayer as described previously[12,27]. Briefly, podocytes (3×10^5) were seeded onto the collagen-coated transwell filters (3 μm pore; Corning, New York, NY) in the top chamber and cultured under differentiating conditions. After 4 days, podocytes were serum-starved overnight and treated for 6 hours with individual plasma samples from normal subjects ($n = 3$) and with plasma from the same diabetic patients before ($n = 3$) or after treatment with GTP ($n = 3$). Cells were washed twice with phosphate buffered saline (PBS) supplemented with 1 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$ to preserve the cadherin-based junctions. The top chamber was then refilled with 0.5 mL of RPMI 1640 and the bottom chamber with 1 mL of RPMI 1640 supplemented with 40 mg/mL of bovine serum albumin and incubated at 37 °C. A small aliquot of media from the top chamber was collected as a single sample 6 hours later and the albumin concentration was determined using a bicinchoninic acid protein assay kit (Sigma).

Statistical analysis. Inaccordancewith apreviousstrial of similardesign[28], wecalculatedthataminimum of19 patients in each group was necessary to detect a 30% change in UACR($\alpha = 0.05, \beta = 0.20$)[29,30]. Data were analyzed using an intention-to-treat principle, defined as participantswhometallof the inclusion criteria, metnoneofthe exclusioncriteria,had atleastonedose ofthestudydrug, andfinallaboratoryassessmentdataavailableforanalysis. Clinicalparametersarepresentedasmediansandinterquartilerangesunlessotherwisenoted.Exploratorydataanalysiswasperformedthroughsummarymeasuresof categoricaldataanddescriptivestatisticsof quantitativedata. To compare categorical clinical variables between groups, the chi-square test was applied and, when necessary, Fisher's exact test. The Mann–Whitney test was performed for numerical comparison of baseline clinical variables between groupsandfrombaselinetotheend of thestudy.Theprimaryoutcomeforalbuminuriaispresentedasthegeometric mean of % change from baseline and two-sided 95% confidence intervals. The within-group geometric mean change (%) is derived by 100x (exp(LS mean change) −1, and the same transformation is applied on the 95% confidence intervalforthegeometricmeanchange(%). Forthechangefrombaseline in theurinaryalbumin-to-creatinineratio (and associated 95% CI), values were back-transformed to provide GTP-to-placebo ratios. *In vitro* data presented as mean ± SD were analyzed with a one-way analysis of variance (ANOVA) followed by Bonferroni test. The significance level adopted for this studywas $p < 0.05$. Statistical analyses were conducted using SAS (Statistical Analysis System) software for Windows (version 9.4, SAS Institute Inc., 2002–2008, Cary, NC, USA).

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Acknowledgements

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant 2008/57560-0 and 2014/22687-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grant 304026/2013-1). A.P. and D.D.A. received postdoctoral and doctoral scholarships from FAPESP, respectively. We are most grateful to Dr. Otavio Berwanger for helping with the design of the study. We are grateful for the technical assistance from personnel at Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, and the Diabetic Nephropathy Clinic, State University of Campinas (UNICAMP). We also thank the personnel from the Cardiology Unit, particularly Dr. Wilson Nadruz, Jr., for assistance with the 24-hour blood pressure monitoring. We are also most grateful to Mr. Paulo Fanti de Oliveira and Cleide A. Moreira Silva for helping with statistical analyses.

Author Contributions

C.M.B. acquired data, drafted the manuscript, and approved the final version. A.P. acquired data, revised the manuscript, and approved the final version. D.A.D. acquired data, revised the manuscript, and approved the final version. J.M.L.d.F. played an important role in interpreting the results, revised the manuscript, and approved the final version. J.B.L.F. conceived and designed the work that led to this submission, interpreted the results, revised the manuscript, and approved the final version.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Borges, C. M. *et al.* The use of green tea polyphenols for treating residual albuminuria in diabetic nephropathy: A double-blind randomised clinical trial. *Sci. Rep.* **6**, 28282; doi: 10.1038/srep28282 (2016).

Artigo 2: *Metformin arrests the progression of established kidney disease in the subtotal nephrectomy model of chronic kidney disease.*

Am J Physiol Renal Physiol 318: F1229 –F1236, 2020.
First published April 6, 2020; doi:10.1152/ajprenal.00539.2019.

RESEARCH ARTICLE

Metformin arrests the progression of established kidney disease in the subtotal nephrectomy model of chronic kidney disease

Cynthia M. Borges,[1] Clarice Kazue Fujihara,[2] Denise M. A. C. Malheiros,[3] Victor Ferreira de Ávila,[2] Guilherme Pedrom Formigari,[1] and José B. Lopes de Faria[1]

[1]*Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, Faculty of Medical Sciences, State University of Campinas, Campinas, São Paulo, Brazil;* [2]*Faculty of Medicine, Renal Division, Department of Clinical Medicine, University of São Paulo, São Paulo, Brazil; and* [3]*Faculty of Medicine, Renal Pathology, Department of Pathology, University of São Paulo, São Paulo, Brazil*

Submitted 15 November 2019; accepted in final form 18 March 2020

Borges CM, Fujihara CK, Malheiros DM, de Ávila VF, Formigari GP, Lopes de Faria JB. Metformin arrests the progression of established kidney disease in the subtotal nephrectomy model of chronic kidney disease. *Am J Physiol Renal Physiol* 318: F1229 –F1236, 2020. First published April 6, 2020; doi:10.1152/ajprenal.00539.2019.—Metformin, an AMP-activated protein kinase (AMPK) activator, has been shown in previous studies to reduce kidney fibrosis in different models of experimental chronic kidney disease (CKD). However, in all of these studies, the administration of metformin was initiated before the establishment of renal disease, which is a condition that does not typically occur in clinical settings. The aim of the present study was to investigate whether the administration of metformin could arrest the progression of established renal disease in a well-recognized model of CKD, the subtotal kidney nephrectomy (Nx) model. Adult male Munich-Wistar rats underwent either Nx or sham operations. After the surgery (30 days), Nx rats that had systolic blood pressures of >170 mmHg and albuminuria levels of >40 mg/24 h were randomized to a no-treatment condition or to a treatment condition with metformin (300 $mg \cdot kg^{-1} \cdot day^{-1}$) for a period of either 60 or 120 days. After 60 days of treatment, we did not observe any differences in kidney disease parameters between Nx metformin-treated and untreated rats. However, after 120 days, Nx rats that had been treated with metformin displayed significant reductions in albuminuria levels and in markers of renal fibrosis. These effects were independent of any other effects on blood pressure or glycemia. In addition, treatment with metformin was also able to activate kidney AMPK and therefore improve mitochondrial biogenesis. It was concluded that metformin can arrest the progression of established kidney disease in the Nx model, likely via the activation of AMPK.

AMP-activated protein kinase; chronic kidney disease; fibrosis; metformin; subtotal nephrectomy

INTRODUCTION

Chronic kidney disease (CKD) is a public health problem that affects ~11–13% of people worldwide (15). Regardless of the disorder that led to CKD, patients with CKD have increased morbidity and mortality, especially in end-stage renal disease (34). The current therapies available for CKD, which include glycemic, lipid, and blood pressure control associated with angiotensin receptor blockers or angiotensin-converting enzyme inhibitors, are effective. However, these therapies are not sufficient, since many patients still require renal dialysis or are waiting for kidney transplantation (32).

Renal fibrosis is a histopathological hallmark abnormality observed in the late stages of CKD, independent of the initial insult. Recently, a reduction in AMP-activated protein kinase (AMPK) activity has been proposed as an underlying mechanism of renal fibrosis in different experimental models of CKD (28). AMPK is the major energy sensor of most eukaryotic cells, is responsible for controlling cellular metabolism, and represents an important link between metabolic disease and the development of CKD (7). AMPK is activated when there is an increase in the AMP-to-ATP (AMP/ATP) ratio that leads to a decrease in anabolic pathways and an increase in catabolic pathways, thus restoring the cell's energy status (13). However, in addition to its role in cellular energy, AMPK is also involved in many pathophysiological conditions, including renal fibrosis (28). It has been suggested that, in CKD, a reduction in AMPK activity may lead to renal fibrosis through activation of the transforming growth factor-þ (TGF-þ) pathways named Smad2 (25) and Smad3 (23). Concordantly, AMPK activation with drugs such as 5-aminoimidazole-4-carboxamide ribonucleotide or metformin can increase AMPK activity and promote a reduction in the kidney fibrosis process (27).

Metformin is an oral hypoglycemic agent that is considered the firstline treatment for type 2 diabetes mellitus. Metformin activates an upstream kinase, liver kinase B1, which, in turn, regulates the downstream kinase AMPK (29). Metformin also inhibits the mitochondrial respiratory chain, increasing the AMP/ATP ratio and therefore activating AMPK (14). Recently, the potential efficacy of metformin for the prevention of fibrosis has been demonstrated in different organs, including the kidney (27), liver (24), and lung (26). In the kidney, it has been shown that in diabetes mellitus (16), high-fat diet (31), unilateral ureteral obstruction (5), cisplatin (20), and subtotal kidney nephrectomy (Nx) (27) models, metformin was able to ameliorate renal disease and reduce fibrosis. These studies claimed that these effects on kidney protection were mediated by AMPK activation. However, in all these studies, the administration of metformin was initiated before the establishment of renal disease, which is a situation that does not typically occur in clinical settings. In the present study, we investigated

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whether the administration of metformin can prevent the progression of established renal disease (proteinuria and hypertension) in a well-recognized experimental model of CKD, the Nx model.

MATERIALS AND METHODS

Animal model. Sixty-eight adult male Munich-Wistar rats weighing between 220 and 260 g that were at least 8 wk of age were used in this study. All rats were obtained from a local facility of the Faculty of Medicine at the University of São Paulo. All experimental procedures were specifically approved by the local Research Ethics Committee (under Process No. 3856-1) and developed in strict conformity with our institutional guidelines and with international standards for the manipulation and care of laboratory animals. All rats were monitored daily regarding their body weight and general condition. Five-sixths renal ablation (Nx) was performed in a single-step procedure after ventral laparotomy under anesthesia induced with ketamine (50 mg/kg) and xylazine (10 mg/kg im). The right kidney was removed, and two or three branches of the left renal artery were ligated, resulting in the infarction of two-thirds of the left kidney, as previously reported (10).

Sham-operated rats underwent anesthesia and manipulation of the renal pedicles without any removal of renal mass. Postoperatively, all animals received enrofloxacin and, after full recovery, were given free access to tap water, were fed regular rodent chow containing 22% protein (Nuvital Laboratories, Curitiba, Brazil), and were kept at 23 ± 1°C and 60 ± 5% relative air humidity under an artificial 12:12-h light-dark cycle. After renal ablation (30 days), systolic blood pressure (SBP) was determined with an optoelectronic-automated device (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC). Each SBP value was taken as the average of three consecutive measurements obtained after stabilization of the readings. Urinary albumin excretion (UAE) levels were assessed with an ELISA kit (NEPHRAT, Philadelphia, PA). Rats that failed to increase their SBPs above 170 mmHg or failed to increase their UAE levels above 40 mg/day were excluded from the study (n = 3). The remaining 48 Nx rats were divided into the following 2 experimental groups: untreated Nx rats (Nx group; n = 24) and Nx rats that received metformin (300 mg/kg) diluted in drinking water (Nx + Met group; n = 24). Nx rats were distributed in such a way that the initial body weights, SBPs, and albuminuria levels were similar between experimental groups. The treatment was maintained for 60 or 120 days, respectively, with monthly or bimonthly assessments of SBP and UAE taking place. A group of sham-operated rats that received no treatment (sham group; n = 17) was followed concomitantly. At the end of the study, the rats were euthanized, and blood was collected from the right ventricles of their hearts; their renal tissues were also prepared for histomorphometric and immunohistochemical analyses as previously described (8).

Histology. Histopathological changes in the kidneys were assessed using a masked protocol by a single pathologist observer (D. M. A. C. Malheiros). Paraffin-embedded kidney sections were stained with periodic acid-Schiff (PAS) stain to access the percentage of glomeruli with sclerotic lesions using the glomerular sclerosis index (GSI) and stained with Masson's trichrome/sirius red staining to identify interstitial fibrosis.

Glomerular sclerosis index. In 4-μm kidney sections, 100 glomeruli from each rat were examined in a blinded manner. The extent of glomerular injury was estimated by determining the GSI in sections stained by the PAS reaction.

Quantitation of interstitial fibrosis. Kidney sections 4 μm in size were stained with Masson's trichrome and sirius red. In the Masson's trichrome staining procedure, the percentage of the renal cortical area occupied by interstitial tissue was used as a measure of the degree of interstitial fibrosis (%) in 25 consecutive microscopic fields of Masson-stained sections, using a point-counting technique (18) at a final magnification of ×100 on a 144-point grid. For sirius red staining, the percentage of renal fibrosis was quantified as 20 nonoverlapping ×200 fields per animal using the Leica Application Suite (LAS Image Analysis, Leica Microsystems, Buffalo Grove, IL). After the software was set to differentiate the positively stained (red) areas from the negatively stained areas on the first image, the software sequentially opened each image, completed the analysis, stored the data, closed the image, and moved on the next image.

Immunohistochemistry. Immunohistochemistry was performed on 4-mm-thick sections mounted on glass slides that had been precoated with 2% silane. These sections were deparaffinized and rehydrated using conventional techniques, heated in a citrate buffer for antigen retrieval, and then incubated overnight with the primary antibody at 4°C. For the negative controls, incubation with the primary antibody was not performed. The following primary antibodies were used: rabbit monoclonal antibody anti-phosphorylated (p-)Thr^{172} AMPK-α (p-T172AMPK) and anti-phospho-Ser^{79} acetyl-CoA carboxylase (ACC; p-S79ACC) (Cell Signaling Technology, Danvers, MA). Sections were pretreated with 10% hydrogen peroxide in methanol, preincubated with 1% milk to prevent nonspecific binding, and then incubated overnight at 4°C with the primary antibody diluted (p-T172AMPK: 1:10 and p-S79ACC: 1:20) in milk at 1%. After being rinsed with PBS, sections were incubated with a horseradish peroxidase (HRP)-labeled polymer conjugated with secondary antibodies (Dako, Glostrup, Denmark) and then incubated with a 3,3′-diaminobenzidine substrate-chromogen solution (Dako) for development. Renal densities of p-T172AMPK and p-S79ACC positivity in the tubulointerstitial area were evaluated using the Leica Application Suite on 20 sequential low-power microscopic fields (×200) for each animal. After the software was set to differentiate the positively stained (brown) areas from the negatively stained areas on the first image, the software sequentially opened each image, completed the analysis, stored the data, closed the image, and moved on to the next image just as it had for the sirius red staining assessment.

Western blot analysis. Kidney cortical tissues were homogenized in a buffer [10 mM Tris (pH 8), 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 150 mM sodium chloride, 0.1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride] supplemented with a protease inhibitor cocktail (Complete, Boehringer-Mannheim, Indianapolis, IN). The Western blots were performed as previously described (21). The Bradford method was used for protein quantification (4). Proteins were separated on 8–10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The electrophoresis was performed separately and in parallel with kidney lysates that would subsequently be used to detect p-AMPK or total AMPK and p-ACC or total ACC. After being blocked with 5% nonfat milk, membranes were incubated overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-peroxisome proliferator-activated receptor-μ coactivator-1α (PGC-1α; 1:250, Cell Signaling Technology), rabbit monoclonal anti-p-AMPKα (Thr^{172}, 1:1,000, Cell Signaling Technology), rabbit polyclonal anti-AMPKα (1:1,000, Cell Signaling Technology), rabbit polyclonal anti-p-ACC (Ser^{79}, 1:1,000, Cell Signaling Technology), rabbit monoclonal anti-ACC (1:1,000, Cell Signaling Technology), and mouse cocktail anti-total OXPHOS (1:1,000, ab110413, Abcam). Molecular weight markers (Page Ruler; Life Sciences) were used as standards. After being washed with PBS containing 0.1% Tween 20, HRP-linked goat anti-mouse and goat anti-rabbit IgG secondary antibodies were added at a dilution ratio of 1:2,000, and membranes were incubated at room temperature for 1 h. Immunoreactive bands were visualized using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System, Pierce, Rockford, IL). The uniformity of protein loading and transfer efficiency were assessed by reprobing the membranes for vinculin (rabbit monoclonal anti-vinculin antibody, 1:1,000, Cell Signaling Technology). Membranes were scanned with a digital photo documentator (ImageQuant LAS 500, GE, Boston, MA) and analyzed quantitatively

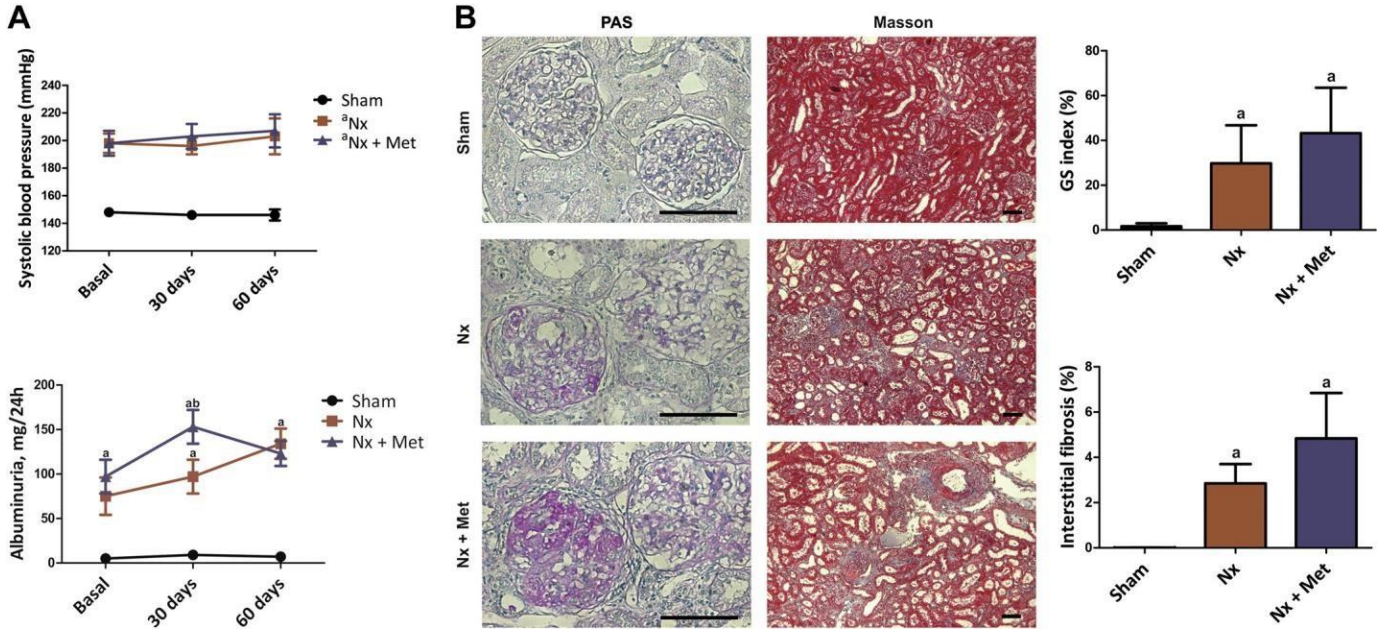

Fig. 1. *A*: systolic blood pressure (mmHg) and serum albumin levels (mg/24 h) during 60 days of treatment. $n = 7–10$. *B*: periodic acid-Schiff (PAS; magnification: ×400) staining was used to analyze glomerular injury and the glomerular sclerosis (GS) index (%). Masson's trichrome (magnification: ×100) was used to evaluate the percentage of renal interstitial fibrosis present after 60 days of treatment. Representative photomicrographs and graphic quantification are shown. Results are expressed as means ± SE; $n = 5–7$. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. $^{a}P < 0.05$ vs. the sham group; $^{b}P < 0.05$ vs. the Nx group. Scale bars = 100 μm.

using ImageJ Software (National Institutes of Health, Bethesda, MD). At least three independent experiments were carried out.

Statistical analysis. Differences among groups were assessed using one-way ANOVA with pairwise posttest comparisons according to the Fisher method. The results are presented as means ± SE. A Spearman's rank correlation was used to estimate the correlation between p-T172AMPK expression and kidney fibrosis (immunohistochemistry of sirius red) and between p-T172AMPK and p-S79ACC expression in renal tissue. Calculations were performed using Statview 5.0 software. *P* values of <0.05 were considered statistically significant.

RESULTS

Sixty days of treatment. The time course of hypertension and albuminuria is shown in Fig. 1*A*. SBP and albuminuria were elevated in the Nx and Nx + Met groups throughout the entire study. Body weights, left kidney weights, serum creatinine levels, and random blood glucose levels are shown in Table 1. Body growth was limited in all Nx groups compared with the sham group. Left kidney weights were diminished in the Nx groups relative to the sham group, although left kidney weights were significantly higher in the Nx group treated with metformin than in the untreated Nx group. Serum creatinine was elevated in Nx rats ($P < 0.05$ vs. sham rats), and treatment with metformin had no effects on serum creatinine levels. Blood glucose concentrations were similar across all groups.

Table 1. *Renal and systemic parameters after 60 days of treatment*

	n	Body Weight, g	Left Kidney Weight, g	Serum Creatinine, mg/dL	Random Blood Glucose, mg/dL
Sham	7	328 ± 2	1.5 ± 0.2	0.4 ± 0.02	153 ± 3.6
Nx	8	297 ± 4	0.4 ± 0.1*	1.06 ± 0.1*	155 ± 15.9
Nx + Met	10	264 ± 19*	0.9 ± 0.1*†	1.19 ± 0.1*	152 ± 7.5

Results are expressed as means ± SE; *n*, number of animals. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. *$P < 0.05$ vs. the sham group; †$P < 0.05$ vs. the Nx group.

Figure 1*B* shows the glomerular changes visible with PAS staining (areas in pink) and also shows renal interstitial fibrosis revealed by Masson's trichrome staining (areas in blue). GSI and the amount of interstitial fibrosis were greater in the Nx groups than in the sham group and were not modified by metformin treatment.

We could not detect any effects of the 60-day metformin treatment on established renal disease of Nx, but we did notice a descending curve tendency in metformin-treated rats and an ascending curve tendency in untreated rats for albuminuria levels (Fig. 1*A*). Therefore, we decided to extend the treatment with metformin for an additional period of 60 days.

Treatment lasting 120 days. SBP and albuminuria levels were markedly elevated in the Nx group throughout the entire study (Fig. 2*A*). After 60 days of treatment, serum albumin levels and SBPs were still not influenced by treatment with metformin. After 120 days, the level of albuminuria was 178 ± 13 mg/24 h in the Nx group and had decreased to 69 ± 8 mg/24 h in the Nx + Met group ($P < 0.0001$). This effect of metformin on albuminuria levels was independent of any changes in blood pressure or blood glucose.

Body growth was also limited in all Nx groups compared with the sham group. Left kidney weights were slightly diminished in the Nx groups relative to the sham group, indicating marked renal hypertrophy given the magnitude of the original renal ablation. Serum creatinine was markedly elevated in Nx rats ($P < 0.05$ vs. sham rats), and metformin treatment had no effects on serum creatinine levels. Once again, blood glucose concentrations were similar across all groups (Table 2).

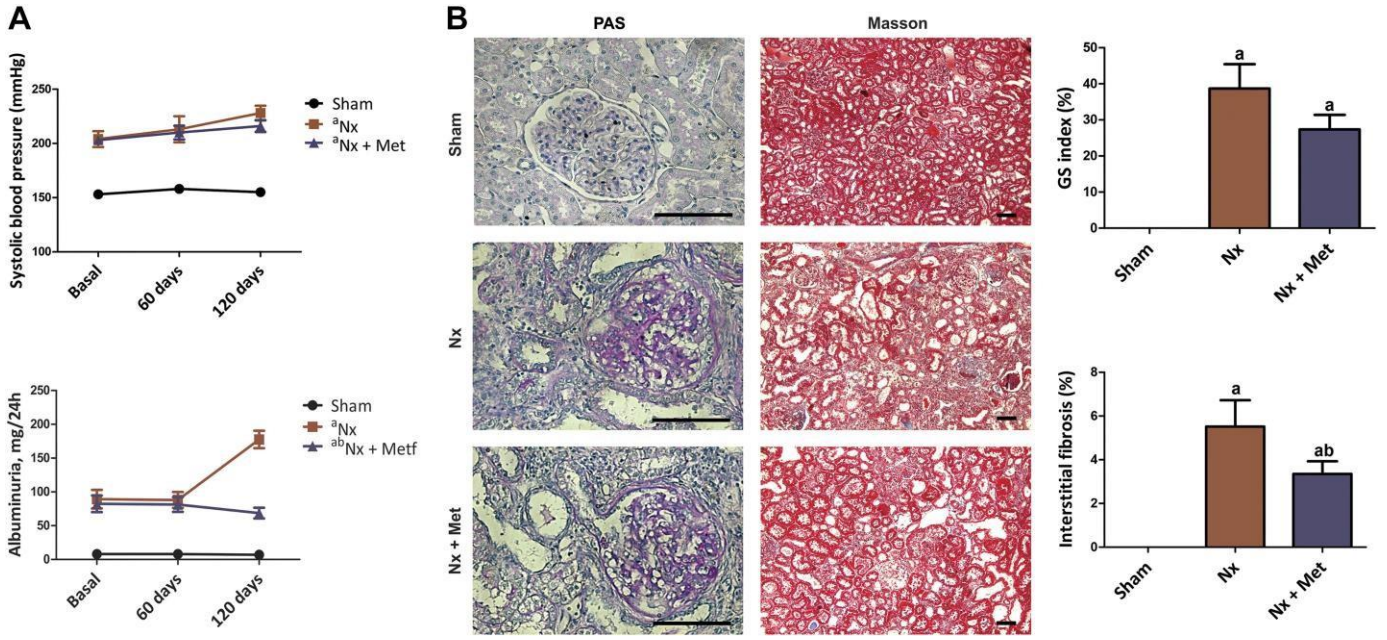

Fig. 2. *A*: systolic blood pressure (mmHg) and albuminuria levels (mg/24 h) during 120 days of treatment. $n = 7–10$. *B*: periodic acid-Schiff (PAS; magnification: ×400) staining was used to analyze glomerular injury and the glomerular sclerosis (GS) index (%). Masson's trichrome (magnification: ×100) was used to evaluate the percentage of renal interstitial fibrosis present after 120 days of treatment. Representative photomicrographs and graphic quantification are shown. Results are expressed as means ± SE; , $n = 6–10$. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. $^{a}P < 0.05$ vs. the sham group; $^{b}P < 0.05$ vs. the Nx group. Scale bars = 100 μm.

In the untreated Nx group, almost 40% of glomeruli exhibited sclerotic lesions. Metformin treatment attenuated glomerular sclerosis, albeit insignificantly, compared with the untreated Nx group. Interstitial renal fibrosis was reduced in metformin-treated rats compared with untreated rats ($P = 0.042$; Fig. 2*B*). Sirius red staining, which is specific to collagen fibers (areas in red), confirmed the changes observed with Masson's trichrome staining ($P = 0.012$; see Fig. 4*A*).

p-T172AMPK, total AMPK, p-S79ACC, and total ACC renal expression. Kidney homogenates from the Nx group had significantly lower levels of p-T172AMPK, p-S79ACC, total AMPK, and total ACC compared with levels of sham rats ($P < 0.0001$), which were partially reversed by metformin treatment (p-T172AMPK: $P < 0.0001$, total AMPK: $P = 0.0007$, p-S79ACC $P = 0.002$, and total ACC: $P = 0.004$, Nx × Nx + Met; Fig. 3). Immunohistochemistry (Fig. 4*A*) confirmed that metformin increases the renal expression of p-T172AMPK and p-S79ACC relative to that demonstrated by untreated Nx rats ($P = 0.0303$), and we observed an inverse correlation between p-T172AMPK expression and interstitial fibrosis ($r = —0.79$, $P = 0.0037$; Fig. 4*B*) and a positive correlation between p-T172AMPK expression and p-S79ACC expression ($r = 0.95$, $P = 0.001$; Fig. 4*B*).

Mitochondrial parameters. PGC-1a expression was significantly ($P = 0.039$) higher in Nx + Met rats than it was in untreated Nx rats (Fig. 5). Complexes I and IV of the kidney mitochondrial respiratory chain were significantly lower in Nx rats, and treatment with metformin had no effect on complexes I–V of the mitochondrial respiratory chain (Fig. 5).

Table 2. *Renal and systemic parameters after 120 days of treatment*

	Body Weight, g	Left Kidney Weight, g	Serum Creatinine, mg/dL	Random Blood Glucose, mg/dL
Sham	358 ± 11	1.2 ± 0.05	0.47 ± 0.08	153 ± 6.6
Nx	314 ± 8*	1.06 ± 0.03*	1.54 ± 0.15*	150 ± 5.4
Nx + Met	283 ± 8*	1.06 ± 0.04*	1.32 ± 0.11*	153 ± 5.6

Results are expressed as means ± SE; $n = 10$ animals/group. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. *$P < 0.05$ vs. the sham group.

DISCUSSION

Preclinical studies comprise an important step in assessing the potential efficacy of drugs for the treatment of human disease. One shortcoming of most of these studies is that they deal with the prevention, not treatment, of disease; this is a problem because the treatment under investigation is usually started at the same time that the disease begins, a situation that rarely occurs in a clinical setting. Here, we showed that metformin was effective at arresting the progression of renal disease in the Nx model, a well-established animal model of progressive CKD. These effects were independent of any effects of metformin on blood pressure or glycemia. Importantly, in the present study, treatment with metformin was initiated after the establishment of CKD, which was characterized by elevated blood pressure and higher albuminuria levels. Of note, a longer period of treatment (120 days) was required to observe the nephroprotective effect of metformin, which was not apparent within 60 days of treatment. This last observation was unsurprising since it is reasonable to expect that a longer period of treatment will be required if treatment is initiated after the disease is already established.

In a previous study, Satriano and collaborators (27) aimed to investigate the contribution of AMPK activity to the early

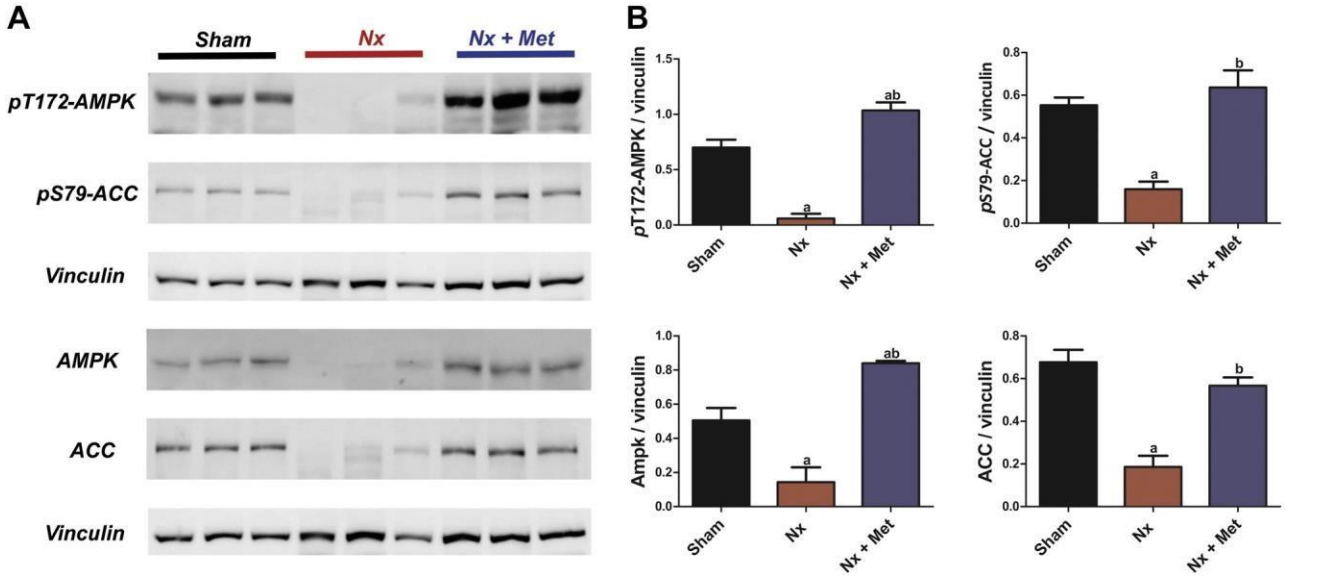

Fig. 3. *A*: Western blot from kidney tissue showing the expression of phosphorylated Thr[172] (pT172-)AMP-activated protein kinase (AMPK), phosphorylated Ser[79] (pS79-)acetyl-CoA carboxylase, AMPK, and ACC in all groups after 120 days of treatment. Blots are representative of three independent experiments. *B*: data quantified from the immunoblots in *A* ($n = 3$). Protein expression levels were normalized to vinculin. Results are expressed as means ± SE. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. [a]$P < 0.05$ vs. the sham group; [b]$P < 0.05$ vs. the Nx group.

pathophysiological alterations observed in the same model used in the present study: the Nx model. Indeed, the authors demonstrated that AMPK activity was reduced as early as 5 days after the kidney ablation and infarction and that this alteration was associated with the subsequent development of progressive renal disease. Importantly, treatment with metformin for 30 days, initiated on the day of the ablation and infarction surgery, was able to ameliorate losses in kidney function as well as kidney fibrosis. Unfortunately, AMPK activity was not reported after 30 days of metformin treatment, but it was shown that, after 7 days of metformin treatment, an elevation in AMPK activity, as assessed using p-172AMPK and p-ACC levels, was observed. The findings of the present study confirmed and extended the observations of Satriano and

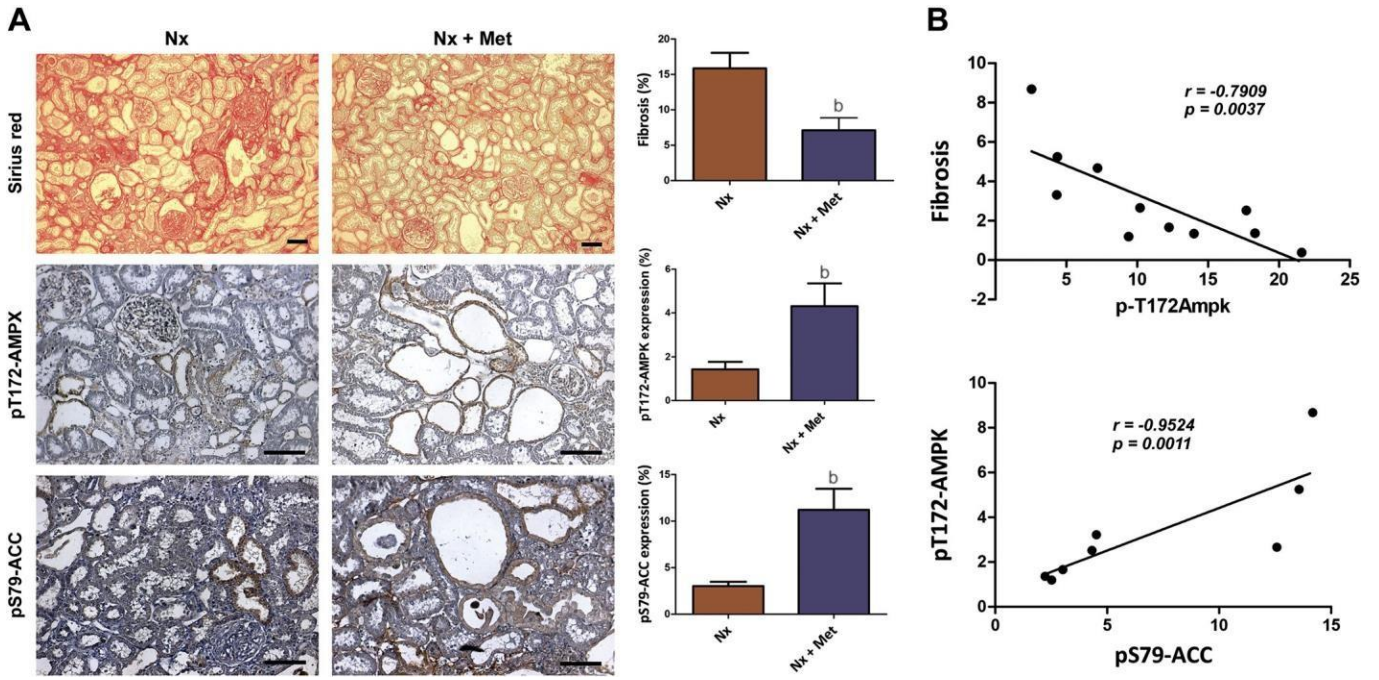

Fig. 4. *A*: representative photomicrographs of renal fibrosis stained with sirius red (magnification: ×100) and immunohistochemistry staining for phosphorylated Thr[172] (pT172-)AMP-activated protein kinase (AMPK) and phosphorylated Ser[79] (pS79-)acetyl-CoA carboxylase (magnification: ×200) in the kidneys of the nephrectomized (Nx) group after 120 days of treatment (magnification: ×200) as well as graphic quantification. $n = 4$–6. *B*: Spearman's rank correlation of fibrosis versus pT172-AMPK and pT172-AMPK versus pS79-ACC expression in the untreated Nx and Nx + metformin (Met) groups. Results are expressed as means ± SE; $n = 4$–6. Sham, sham-operated group. [a]$P < 0.05$ vs. the sham group; [b]$P < 0.05$ vs. the Nx group. Scale bars = 100 μm.

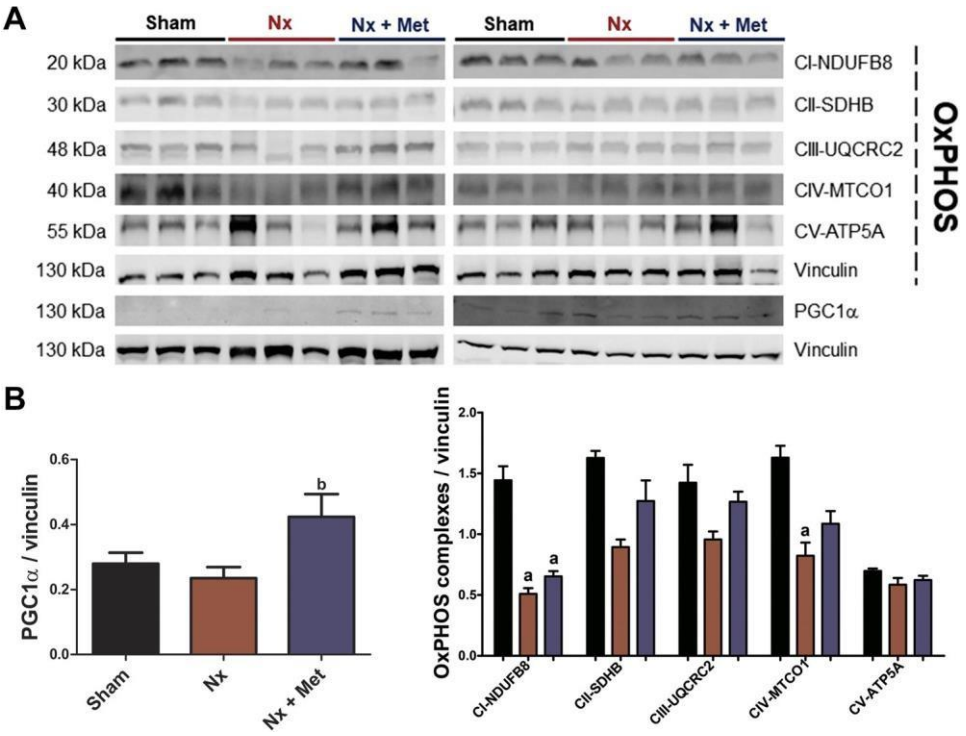

Fig. 5. Western blots from kidney tissue showing the expression of OXPHOS proteins of complexes I–V (CI–CV) and peroxisome proliferator-activated receptor-μ coactivator-1a (PGC-1a). *A*: blots are representative of three independent experiments. *B*: data quantified from the immunoblots in *A*. Protein expression levels were normalized to vinculin. Sham, sham-operated group; Nx, 5/6 nephrectomy; Met, metformin. [a]$P < 0.05$ vs. the sham group; [b]$P < 0.05$ vs. the Nx group.

collaborators. We have shown that the expression of p-T172AMPK, which is indicative of AMPK activity, was diminished in the kidneys of Nx rats compared with control rats and was normalized in metformin-treated rats after 120 days of treatment. More importantly, metformin treatment also elevated the phosphorylation of p-S79ACC, an important target of AMPK that is widely used as a marker of AMPK activation (12). The increase in p-T172AMPK and p-S79ACC induced by metformin was closely and inversely correlated with the magnitude of kidney fibrosis as assessed by sirius red staining. These observations suggest that AMPK activation by metformin may have contributed to the reduction of kidney fibrosis in Nx rats. Another recent study that used a bleomycin model of lung fibrosis demonstrated that AMPK activity was reduced specifically in the lung fibrosis area and that treatment with metformin was able to reverse the established fibrosis associated with an elevation in AMPK activity (26).

In addition to the role of metformin in activating AMPK, this drug has also been suggested to modulate mitochondrial function (11). In the present study, we observed that the administration of metformin to Nx rats promoted an elevation in the expression of PGC-1a, a transcriptional coactivator that is a central inducer of mitochondria biogenesis in cells (2). These observations are important because AMPK has been reported to phosphorylate PGC-1a both in vitro and in vivo (17). In addition, it has been suggested that a reduction of PGC-1a is implicated in the development of renal fibrosis (22). In the present study, the contribution of the elevation in PGC-1a to reducing kidney fibrosis in Nx rats that received metformin remains to be determined. Because the use of metformin may also block complex I of the mitochondrial respiratory chain (11, 14), the present study assessed the expression of proteins involved in the mitochondrial respiratory chain. Although we observed reductions in complexes I and IV in the kidneys of Nx rats, treatment with metformin did not modify the expression of any protein related to the mitochondrial respiratory complex chain. It has been shown that the inhibitory effect of metformin on the mitochondrial respiratory chain requires suprapharmacological doses of metformin (33). We observed an insignificant reduction in body weight in the rats that received metformin. These data are consistent with clinical observations of body weight loss in patients with type 2 diabetes mellitus treated with metformin (1, 19). However, we cannot rule out the possibility that some gastrointestinal side effects of metformin use occurred, which may have affected the lower body weight in metformin-treated rats. Another limitation of the present study was the use of only male rats in the experimental protocol. Therefore, caution must be exercised when extrapolating the data to a female population.

In the present study, metformin was able to reduce albuminuria levels. Although we have not investigated metformin's mechanism of albuminuria reduction, some considerations are of interest. Increased albuminuria levels are frequently observed in patients with CKD. Accordingly, the Nx model has also shown elevated levels of albuminuria (10). As expected, previous studies have implicated podocyte dysfunction as an underlying mechanism for elevated albuminuria levels in the Nx model (3, 30). Although the contribution of AMPK activity to podocyte injury was not investigated in these studies, it has been shown that in vitro podocytes exposed to renal angiotensin II displayed reductions in p-T172AMPK that could be prevented by metformin (6). The expression of renal angiotensin II was elevated in the kidneys of rats that had undergone Nx (9). Therefore, it is possible that increased AMPK activity in podocytes resulting from metformin use may have contributed to improved podocyte function and, consequently, the reduction in albuminuria levels in the present study.

In summary, the present study demonstrated that the use of metformin following the establishment of renal disease in an Nx model can arrest the progression of CKD, including kidney fibrosis. It is possible that the activation of AMPK may contribute to the nephroprotective effect of metformin.

ACKNOWLEDGMENTS

We thank Antonio Ramos Calixto, Diego Andreazzi Duarte, and Ester Mendes for technical assistance.

GRANTS

This work was supported by a grant from the Fundação de Amparo a` Pesquisa do Estado de São Paulo (2014/22687-0). J. B. Lopes de Faria is also the recipient of an award from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (301401/2017-9), which partially funded this research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.M.B., C.K.F., V.F.A., and G.P.F. performed experiments; C.M.B., C.K.F., G.P.F., and J.B.L.F. interpreted results of experiments; C.M.B. and G.P.F. prepared figures; C.M.B. drafted manuscript; C.M.B., C.K.F., D.M.A.C.M., V.F.A., and J.B.L.F. approved final version of manuscript; D.M.A.C.M., G.P.F., and J.B.L.F. analyzed data; J.B.L.F. conceived and designed research; J.B.L.F. edited and revised manuscript.

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4. DISCUSSÃO

Na presente tese, constatamos que o emprego da epigalocatequina galato é útil no tratamento de pacientes com nefropatia diabética, reduzindo a albuminúria por reduzir a apoptose de podócitos. Além disso, demonstramos que a metformina é eficaz em reduzir a progressão da doença renal crônica já estabelecida, por ativar a AMPK e consequentemente reduzir a fibrose renal.

No artigo 1, confirmamos (em um contexto clínico) os resultados do nosso estudo pré-clínico. Nele foi sugerido que a epigalocatequina galato é capaz de reduzir a albuminúria na nefropatia diabética devido, provavelmente, ao aumento na ativação da via Wnt e consequente redução da apoptose de podócitos (Peixoto et al., 2015). No presente estudo, demonstramos que a adição de Polifenon E (epigalocatequina galato) à doses máximas de iECA e/ou BRA é segura e eficaz na redução da albuminúria residual em pacientes com nefropatia diabética. E o possível mecanismo envolvido nesse efeito do Polifenon E se deve à redução da apoptose de podócitos que resulta em uma melhor seletividade na barreira glomerular.

A diminuição nos níveis de DKK-1 (um inibidor da via Wnt) e, consequente, maior ativação da via Wnt, parece explicar a redução da apoptose podocitária; já que observamos uma redução no nível plasmático do DKK-1 nos pacientes tratados com Polifenon E, comparados ao grupo que recebeu placebo. Além disso, confirmamos no estudo *in vitro*, com cultura de podócitos humanos, que o plasma de pacientes diabéticos com nefropatia promove apoptose de podócitos, conforme avaliado pela caspase clivada 3 e pelo teste TUNEL. Tal ação ocorre, provavelmente, devido a um aumento da concentração de DKK-1 no plasma. Essa sugestão é apoiada por nossa observação de que o efeito protetor do plasma de pacientes diabéticos que receberam GTP foi perdido quando tratamos os podócitos com DKK-1. Resultados semelhantes também foram observados na permeabilidade à albumina no estudo *in vitro*.

No artigo 2, verificamos que a metformina foi capaz de interromper a progressão da doença renal em ratos submetidos à nefrectomia subtotal 5/6 - modelo animal bem estabelecido de DRC progressiva. Estes efeitos foram

independentes de quaisquer efeitos da metformina na pressão sanguínea arterial ou na glicemia. É importante ressaltar que, no nosso estudo, o tratamento com metformina foi iniciado após o estabelecimento da DRC, caracterizada por pressão arterial elevada e níveis mais altos de albuminúria; complementando os achados do estudo prévio de Satriano e colaboradores (Satriano et al., 2013), no qual a metformina também reduziu a fibrose renal. Porém, no referido estudo, o tratamento foi iniciado no dia da nefrectomia e durou apenas 30 dias.

Demonstramos que a expressão de p-T172AMPK (forma ativada da AMPK) diminuiu nos rins de ratos Nx em comparação com ratos controle e foi normalizada em ratos tratados com metformina após 120 dias de tratamento. O tratamento com metformina também elevou a fosforilação do p-S79ACC - um importante alvo da AMPK, utilizado como marcador da ativação da AMPK (Hardie et al., 2012). O aumento de p-T172AMPK e p-S79ACC induzido pela metformina correlacionou-se inversamente com a intensidade de fibrose renal (coloração sirius red). Essas observações sugerem que a ativação da AMPK pela metformina pode ter contribuído para a redução da fibrose renal em ratos Nx.

Estudos anteriores *in vitro* e *in vivo* relataram que a AMPK fosforila o PGC-1α (Jäger et al., 2007) - principal coativador transcricional da biogênese mitocondrial (Austin et al., 2012) - e que a redução de PGC-1α está envolvida no desenvolvimento de fibrose renal (Lynch et al., 2018). Nossos resultados mostram que a administração de metformina em ratos Nx aumentou a expressão do PGC-1α. Como o uso de metformina também pode bloquear o complexo I da cadeia respiratória mitocondrial (Glossmann and Lutz, 2019; Herzig and Shaw, 2018), o presente estudo avaliou a expressão de proteínas envolvidas nesta cadeia. Embora tenhamos observado reduções nos complexos I e IV nos rins de ratos Nx, o tratamento com metformina não modificou a expressão das proteínas relacionadas à cadeia do complexo respiratório mitocondrial, provavelmente, porque o efeito inibitório da metformina nesta cadeia requer doses suprafarmacológicas desta substância (Wang et al., 2019).

5. CONCLUSÃO

- Os resultados do presente estudo demonstram que o tratamento com Polifenon E (epigalocatequina galato) associado aos inibidores de SRA é capaz de reduzir a albuminúria de pacientes com nefropatia diabética.

- O efeito nefroprotetor observado pode ocorrer devido à capacidade do Polifenon E de reduzir a apoptose de podócitos como resultado da ativação da via Wnt; já que o Polifenon E reduz o nível sérico de DKK1 (um inibidor da via Wnt) dos pacientes.

- O tratamento com metformina é capaz de reduzir a fibrose renal em ratos à nefrectomia subtotal 5/6 e doença renal estabelecida (proteinúria e hipertensão arterial sistêmica). É possível que a ativação da AMPK contribua para o efeito nefroprotetor da metformina.

6. PERSPECTIVAS

Nossos resultados evidenciam que, tanto o Polifenon E (epigalocatequina galato), quanto a metformina são medicamentos nefroprotetores nas situações estudadas e que portanto têm o potencial de serem adicionados ao tratamento dos pacientes com DRC; especialmente naqueles que respondem parcialmente ao tratamento atual. Entretanto, mais estudos se fazem necessários para que o Polifenon E e metformina sejam recomendados com segurança no tratamento destes pacientes. Assim, sugere-se realizar:

1. Estudo randomizado multicêntrico com amostras maiores de pacientes para confirmar a eficácia e segurança a longo prazo da adição do Polifenon E (epigalocatequina galato) aos inibidores do SRA, com o objetivo de minimizar não só a albuminúria mas também a progressão da ND.
2. Estudo clínico randomizado em pacientes com DRC não-diabética afim de testar a eficácia do uso da metformina na proteção da função renal.

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8. APÊNDICE

Artigo 3: Theobromine increases NAD+/Sirt-1 activity and protects the kidney under diabetic conditions

Am J Physiol Renal Physiol 308: F209 –F225, 2015.
First published November 19, 2014; doi:10.1152/ajprenal.00252.2014.

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Theobromine increases NAD^+/Sirt-1 activity and protects the kidney under diabetic conditions

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Submitted 9 May 2014; accepted in final form 17 November 2014

Papadimitriou A, Silva KC, Peixoto EB, Borges CM, Lopes de Faria JM, Lopes de Faria JB. Theobromine increases NAD^+/Sirt-1 activity and protects the kidney under diabetic conditions. *Am J Physiol Renal Physiol* 308: F209 –F225, 2015. First published November 19, 2014; doi:10.1152/ajprenal.00252.2014.—Reduction in sirtuin 1 (Sirt-1) is associated with extracellular matrix (ECM) accumulation in the diabetic kidney. Theobromine may reduce kidney ECM accumulation in diabetic rats. In the current study, we aimed to unravel, under diabetic conditions, the mechanism of kidney ECM accumulation induced by a reduction in Sirt-1 and the effect of theobromine in these events. In vitro, we used immortalized human mesangial cells (iHMCs) exposed to high glucose (HG; 30 mM), with or without small interfering RNA for NOX4 and Sirt-1. In vivo, spontaneously hypertensive rats (SHR) were rendered diabetic by means of streptozotocin and studied after 12 wk. The effects of treatment with theobromine were investigated under both conditions. HG leads to a decrease in Sirt-1 activity and NAD^+ levels in iHMCs. Sirt-1 activity could be reestablished by treatment with NAD^+, silencing NOX4, and poly (ADP-ribose) polymerase-1 (PARP-1) blockade, or with theobromine. HG also leads to a low AMP/ATP ratio, acetylation of SMAD3, and increased collagen IV, which is prevented by theobromine. Sirt-1 or AMPK blockade abolished these effects of theobromine. In diabetic SHR, theobromine prevented increases in albuminuria and kidney collagen IV, reduced AMPK, elevated NADPH oxidase activity and PARP-1, and reduced NAD^+ levels and Sirt-1 activity. These results suggest that in diabetes mellitus, Sirt-1 activity is reduced by PARP-1 activation and NAD^+ depletion due to low AMPK, which increases NOX4 expression, leading to ECM accumulation mediated by transforming growth factor (TGF)-þ1 signaling. It is suggested that Sirt-1 activation by theobromine may have therapeutic potential for diabetic nephropathy.

AMPK; diabetic nephropathy; extracellular matrix accumulation; Sirt-1 (sirtuin 1); theobromine

DIABETIC NEPHROPATHY (DN), one of the most serious and common microvascular complications of diabetes, is considered the leading cause of chronic renal failure and primary indication for dialysis and transplantation (11). The identification of new therapeutic targets and compounds that can combat this devastating disease is an urgent matter. Excess amounts of reactive oxygen species (ROS) in diabetes mellitus (DM) can cause increased expression of extracellular matrix (ECM) genes, with progression to fibrosis, proteinuria, and end-stage renal disease (11, 33). In DM, the link between higher levels of ROS and ECM accumulation is multifactorial and may include the reduction of sirtuin 1 [Sirt-1; silent information regulator 2 (Sir2)] activity (16, 27). The Sirt-1 protein is the founding member of a family of NAD^+-dependent deacetylases, which are best known for their acknowledged link to longevity associated with caloric restriction (CR) (7, 12, 16, 29). Sirt-1 activity is regulated via the availability of its cosubstrate, NAD^+ (18, 19). It has been shown that a higher level of ROS promotes activation of the NAD^+-dependent DNA repair enzyme, poly(ADP-ribose) polymerase-1 (PARP-1), with subsequent NAD^+ depletion and downregulation of Sirt-1 activity (37). Whether this mechanism of Sirt-1 reduction is operative in the diabetic kidney is unknown and deserves further investigation. Transforming growth factor-þ1 (TGF-þ1) has a critical role in ECM accumulation in DM (49). Some experimental DM studies have shown that a reduction in Sirt-1 is associated with increased ECM accumulation (30, 41). However, the way in which low Sirt-1 activity contributes to kidney ECM accumulation in DM remains to be determined.

Several investigators (24, 26, 30, 41, 44, 45) have shown that the renal expression/activity of Sirt-1 is diminished in different models of experimental DM, although contradictory results have also been reported (25). Similarly, it has been shown in human kidneys that the expression of Sirt-1, as assessed by immunohistochemistry or mRNA expression, was significantly reduced in patients with DN compared with normal subjects or patients with minimal changes disease (6). The mechanism involved in Sirt-1 reduction in diabetic conditions is not totally clear (27), but a reduction in the phosphorylation of AMP-activated protein kinase (AMPK) may play a role (24). It has been shown that maneuvers that could increase Sirt-1 activity may lead to kidney protection in DM (24, 26, 45, 47).

Several experimental and clinical studies have demonstrated that cocoa may be beneficial to human health under various conditions (22). It is generally accepted that the salutary effects of cocoa are related to its high polyphenol content (22). However, we were surprised by our observation that cocoa with low polyphenol (CL; 0.5% of polyphenol, Table 1 and Fig. 1, *A*–*E*) was as efficient as cocoa enriched with polyphenol (CH; 60% of polyphenol) (36) in reducing ECM accumulation and oxidative stress in the kidneys of diabetic rats. This observation prompted us to investigate which compound in CL could be responsible for that beneficial effect, using ultra-

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Table 1. *Physical and metabolic parameters of experimental groups after 16 wk of diabetes*

Group	Initial Body Weight, g	Final Body Weight, g	HbA1c, %	Kidney Weight/Body Weight, ×100	Systolic Blood Pressure, mmHg
SHR CT	283 ± 17	346 ± 27	5.3 ± 0.28	0.38 ± 0.03	186.1 ± 9.4
SHR DM	280 ± 9	224 ± 59*	13.1 ± 0.37*	0.53 ± 0.09§	181.7 ± 18.8
SHR DM CL	259 ± 26	258 ± 59*	13.0 ± 0.30*	0.53 ± 0.10§	184.7 ± 16.9

Values are means ± SE. SHR, spontaneously hypertensive rats; CT, control rats, $n = 6$; DM, diabetic rats, $n = 8$); DM CL, diabetic rats treated with cocoa low in polyphenols (0.5%, $n = 10$). *$P < 0.0001$ vs. SHR CT group.

performance liquid chromatography (UPLC)-mass spectrometry techniques. Chromatography analysis of components within the CL and CH showed the presence of a common component in both CH and CL. Mass spectrometry analysis in CH and CL showed the presence of a structure with a molar mass of 181 g/mol in the common compound identified. Fragmentation analysis of the structure identified by mass spectrometry following ionization showed the product ion spectra of *m*/*z* 181, which corresponds to theobromine, the nonpolyphenol component. The concentration of theobromine in both CH and CL extracts was estimated at ~8% (data not shown).

Theobromine is a methylxanthine that has been implicated as a contributor to the positive effects of cocoa (1, 23). A recent randomized clinical trial demonstrated that theobromine is safe and enhances HDL cholesterol in healthy volunteers (35). Whether theobromine is renoprotective and has any effect on Sirt-1 activity in DN is unknown.

The aims of the present study were to determine the mechanism of Sirt-1 activity reduction under diabetic conditions, the contribution of a reduction in Sirt-1 to mediation of TGF-β1 signaling and ECM accumulation, and whether theobromine exhibits renoprotective effects through increasing Sirt-1 activity.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Identification and quantification of polyphenols and nonpolyphenols in cocoa extract. The identification of the components of cocoa extract was assessed by HPLC (Waters, Milford, MA) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) (13, 14).

Animals and study design. This study protocol was approved by the local committee for ethics in animal research (CEEA/IB/UNICAMP, protocol no. 1834-1). Male spontaneously hypertensive rats (SHR) supplied by Taconic (Germantown, NY) were used in this study. We chose to induce diabetes in SHR because they present a more progressive form of renal disease (8) and we have a vast amount experience with this model (4, 10, 33, 38), and because of the frequent association of diabetes with hypertension in human diabetic kidney disease. Diabetes was induced in 12-wk-old male SHR via a single intravenous injection of streptozotocin (STZ; 60 mg/kg in sodium citrate buffer, pH 4.5) via the tail vein. Control SHR received only the vehicle (citrate buffer). Rats with plasma glucose concentrations >15 mmol/l were considered diabetic in these experiments. Systolic blood pressure was obtained with tail-cuff plethysmography in nonanesthetized rats using a 229 blood pressure amplifier/pump (ITCC Life Science, Woodland Hills, CA). Forty-eight hours after the STZ injection, the diabetic rats were randomly assigned to receive no treatment or treatment with theobromine (5 $mg \cdot kg^{-1} \cdot day^{-1}$ diluted in drinking water). Although the 8% of theobromine in 24 mg/kg of CL that the SHR received corresponds to 2 mg/kg of theobromine, we chose to treat them with 5 mg/kg. We made this decision because in the recent study in humans, the dose of theobromine was roughly 14 mg/kg (35), and it has been estimated that acute oral toxicity in rats is achieved with a much higher dose (950 mg/kg) (42). Therefore, 5 mg/kg of theobromine to treat rats is still quite a low dose.

During the study, the diabetic rats received 2 U of insulin (human insulin HI-0310; Lilly, Indianapolis, IN) three times per week, subcutaneously. After 12 wk of diabetes, the rats were euthanized by carbon dioxide (CO_2) asphyxiation. Anesthetic procedures were used in full, and all precautions were taken to ensure that the animals did not suffer unduly during and after the experimental procedure. The kidneys were snap-frozen at −80°C for future assays.

Albumin excretion rate. The albumin excretion rate (AER) of a 24-h urine collection was determined with an ELISA kit (Nephrat II; Exocell, Philadelphia, PA) (38).

Renal histopathology. Four-micrometer kidney sections were stained with periodic acid-Schiff (PAS), and matrix mesangial expansion, quantified by Leica Application Suite (LAS Image Analysis, Leica Microsystems, Buffalo Grove, IL), was derived from the assessment of 30 glomeruli from each rat.

Immortalized human mesangial cell culture. Immortalized human mesangial cells (iHMCs) were kindly provided by Dr. Nestor Schor (Dept. of Medicine, Nephrology Div., Federal University of São Paulo, São Paulo, Brazil) and from Dr. Bernhard Banas (Nephrology Center, Medical Policlinic, Ludwig-Maximilian University of Munich, Munich, Germany) (2, 9). The concentrations of treatments used in the high-glucose (HG; 30 mM) medium or TGF-β1 (5 ng/ml) in all experiments were chosen after carrying out a thiazolyl blue tetrazolium bromide (MTT) assay (data not shown). Mannitol was used as an osmotic control for 30 mM D-glucose.

Experimental conditions of iHMCs. iHMCs were kept without serum in normal glucose (NG; 5.5 mM) or HG or TGF-β1 for 24 h in the presence of 44 nM theobromine or 0.5% polyphenols (0.25%

Fig. 1. Cocoa with low polyphenol (CL 0.5%) and theobromine in diabetic spontaneously hypertensive rats (SHR) reduce extracellular matrix accumulation, oxidative stress, albuminuria, and renal hypertrophy, respectively. *A–D*: photomicrographs (*A* and *C*) and quantification (*B* and *D*) of the glomerular periodic acid-Schiff (PAS; *A*)- and collagen IV (*C*)-stained areas from all groups. *A* and *C*: original magnification ×400 counterstained with hematoxylin. Scale bar = 50 μm. Values are means ± SE of 3 independent experiments, and images are representative of 6 rats/group. *$P = 0.0001$ vs. SHR control (CT). *E*: NADPH-dependent reactive oxygen species (ROS) generation in renal cortical homogenate and expressed as relative luminescence units (RLU)/mg protein. Values are means ± SE of 3 independent experiments for 6 rats/group. *$P = 0.013$ vs. CT. ±$P = 0.016$ vs. diabetic SHR (DM). *F*: albumin excretion rate (AER) in control (SHR CT), control treated with theobromine (TB; SHR CT TB), diabetic (SHR DM), and diabetic treated with theobromine (SHR DM TB) rats. Values are means ± variance of 3 independent experiments for 6 rats/group. *$P = 0.025$ vs SHR CT. *G–J*: photomicrographs (*G* and *I*) and quantification (*H* and *J*) of the glomerular PAS (*G*)- and collagen IV (*I*)-stained areas from all groups. *$P < 0.001$ vs SHR CT. *G* and *I*: original magnification ×400 counterstained with hematoxylin. Scale bar = 50 μm. Values are means ± SE of 3 independent experiments, and images are representative of 6 rats/group. *$P < 0.005$ vs. SHR CT.

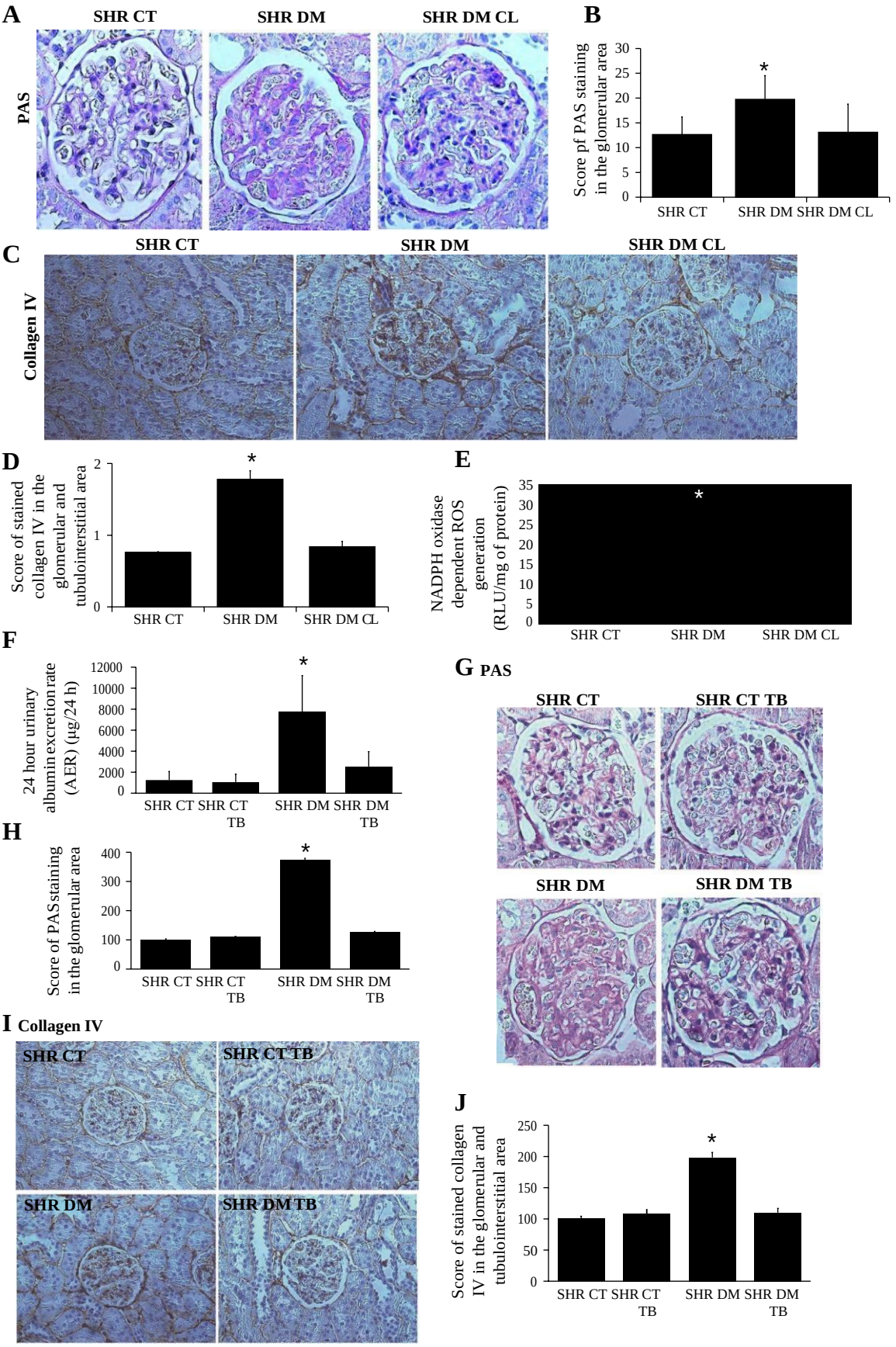


Table 2. *Physical and metabolic parameters of experimental groups following 12 wk of diabetes*

Group	Initial Body Weight, g	Final Body Weight, g	HbA1c, %	Kidney Weight, g	Kidney Weight/Body Weight, ×100	Systolic Blood Pressure, mmHg
SHR CT	260 ± 20	332 ± 19	5.1 ± 0.23	1.33 ± 0.11	0.399 ± 0.078	199.9 ± 9.6
SHR CT TB	270 ± 23	330 ± 18	5.3 ± 0.08	1.19 ± 0.07	0.359 ± 0.04	194.6 ± 11.2
SHR DM	268 ± 18	244 ± 48*	13.7 ± 0.22*	1.61 ± 0.06†	0.674 ± 0.08§	198.6 ± 11.9
SHR DM TB	275 ± 22	271 ± 22*	13.6 ± 0.27*	1.25 ± 0.06	0.448 ± 0.08±	185.2 ± 14.2

Values are means ± SE. CT, control rats, $n = 7$; CT TB, control rats treated with theobromine, $n = 7$; DM, diabetic rats, $n = 7$; DM TB, diabetic rats treated with theobromine, $n = 8$. *$P < 0.0001$ vs. SHR CT group. §$P < 0.005$ vs. SHR CT group. ±$P = 0.016$ vs. SHR DM group. †$P = 0.025$ vs. SHR CT group.

epicatechin and 0.25% catechin), AMPK blocker compound C (CC; 10 μM), PARP-1 activity inhibitor (PJ-34, 1 μM), Sirt-1 activity blocker (EX-527), NAD^+ (300 μM), 100 nM small interfering RNA (siRNA) or scrambled (Scr) for Sirt-1, or 200 nM for NOX4. Theobromine (44 nM) was used as it corresponds to its 8% composition within 100 ng/ml of CL.

Transient transfection with siRNAs. The siRNA duplexes and Src siRNA corresponding to human NOX4 and Sirt-1 were obtained from Invitrogen (Carlsbad, CA) and Santa Cruz Biotechnology (Danvers, MA), respectively. The transient transfection of siRNAs was carried out using Lipofectamine transfection reagent (Invitrogen) (5, 47).

NADPH oxidase activity. NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as previously described, with a few modifications (38).

Western blot and immunoprecipitation analysis. The samples and Western blots were prepared as previously described (4, 10, 38). The following primary antibodies were used: goat anti-type IV collagen (1:500; Southern Biotech, Birmingham, AL), rabbit anti-NOX4 (1:500; Santa Cruz Biotechnology), mouse anti-nitrotyrosine (1:2,000; EMD Millipore, Billerica, MA), mouse anti-PARP-1 (1:1,000; Trevigen, Gaithersburg, MD), rabbit phosphorylated AMPK (Thr^{172}; 1:1,000; Cell Signaling Technology, Danvers, MA), rabbit total AMPK (1:1,000; Cell Signaling Technology), goat anti-fibronectin (1:500; Calbiochem, La Jolla, CA), and rabbit anti-acetyl and anti-SMAD3 (1:500; Cell Signaling Technology). To verify the uniformity of the protein load and transfer efficiency across the test samples, the membranes were reprobed with actin (goat polyclonal anti-actin antibody, diluted 1:1,000; Santa Cruz Biotechnology). For immunoprecipitation analysis, 1,000 μg of total protein in both renal cortex homogenates or iHMC cell lysates were immunoprecipitated with rabbit anti-SMAD3 (Cell Signaling Technology) or rabbit anti-Sirt-1 (Santa Cruz Biotechnology) using protein A agarose beads (Santa Cruz Biotechnology). Precipitates were then analyzed by Western blotting with rabbit anti-lysine (Cell Signaling Technology) and reprobed with rabbit anti-SMAD3 (Cell Signaling Technology) or

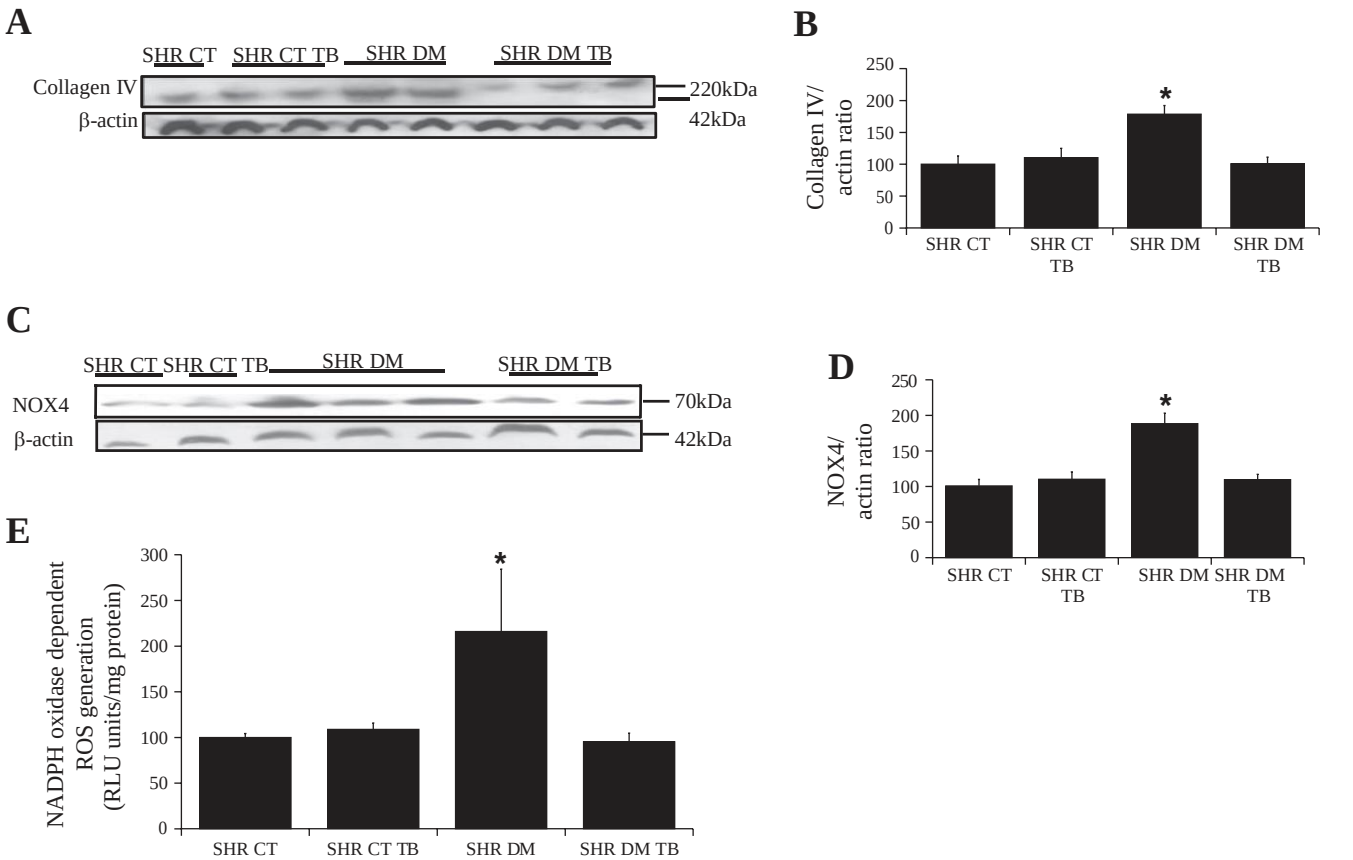


Fig. 2. Theobromine in diabetic SHR prevented ECM accumulation and oxidative stress. *A–D*: Western blot analysis of collagen IV (*A*) and NOX4 (*C*) in the renal cortex of all groups followed by quantification (*B* and *D*) of collagen IV and NOX4/β-actin ratios. *$P = 0.015$ vs. SHR CT for collagen IV. *$P = 0.002$ vs. SHR CT for NOX4. *E*: NADPH-dependent ROS generation in renal cortical homogenate and expressed as RLU/mg protein. Values are means ± SE of 3 independent experiments with 6 rats/group. *$P < 0.0001$ vs. SHR CT.

with rabbit anti-NOX4 (Santa Cruz Biotechnology) or rabbit phosphorylated AMPK (Thr[172]; Cell Signaling Technology).

Immunohistochemistry. To detect ECM accumulation and oxidative stress-induced DNA base modification, immunohistochemistry was carried out for collagen IV and 8-hydroxy-2-deoxyguanosine (8-OHdG; a DNA base-modified product), respectively (4, 38). The primary antibodies used were goat anti-type IV collagen (Southern Biotech) and 1:50 dilution for mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). Collagen IV and nuclear 8-OHdG intensity in both the glomerulus and tubulointerstitial area were quantified using the Leica Application Suite in 50 sequential high-power microscopic fields (×400).

Double immunofluorescence for Sirt-1 and PARP. Kidney halves fixed in 4% paraformaldehyde and frozen in Tissue-Tek O.C.T. compound-embedding medium were autoclaved for 10 min in 120 mmHg. Then, the tissue was permeabilized with 0.3% Triton X-100, and the unspecific sites were blocked by 1% BSA+0.3% Triton X-100. The primary antibodies used were rabbit anti-Sirt-1 diluted 1:10 (Santa Cruz Biotechnology) and mouse anti-PARP-1 diluted 1:10 (Enzo Life Sciences, Farmingdale, NY). Incubation with specific secondary antibodies associated with fluorochromes Alexa 488 or Alexa 594 was performed. The images were observed under confocal microscopy (Leica TCS SP5 II), and the colocalization of stainings was quantified by Image J software.

2-7-Dichlorodihydrofluorescein diacetate measurement of ROS production. Intracellular ROS levels were measured by 2-7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as previously described (10).

Sirt-1 activity quantification. Sirt-1 activity levels in the iHMC lysates (47) and kidney cortexes of the SHR (41) were quantified based on an enzymatic reaction by a fluorometric Sirt-1 assay kit (Fluor-de-Lys Kit; Enzo Life Sciences) using a fluorogenic peptide encompassing residues 379–382 of p53, acetylated on lysine 382. The acetylated lysine residue was coupled to an aminomethylcoumarin

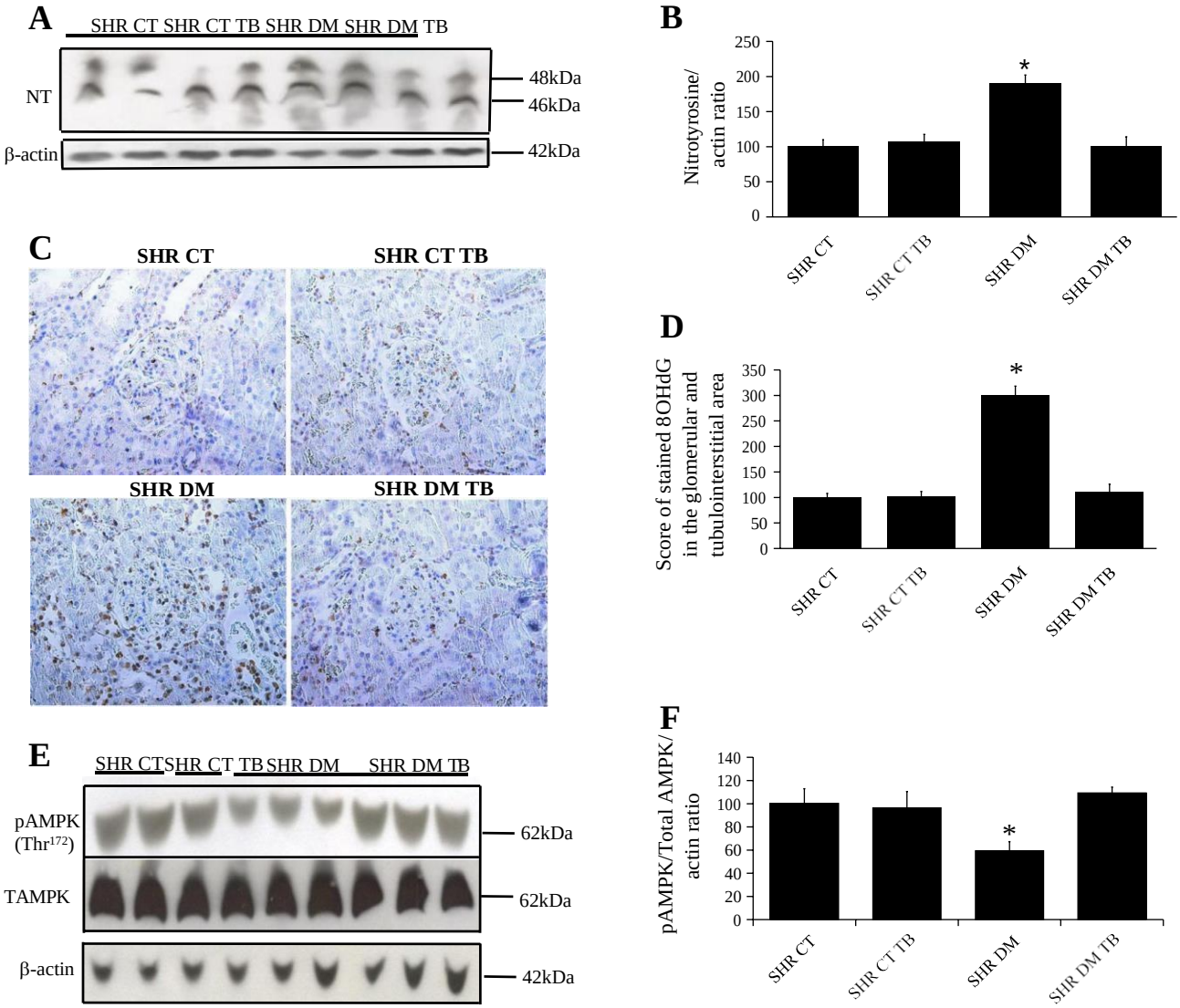


Fig. 3. Theobromine reduces oxidative stress markers in diabetic SHR and activates AMPK. *A* and *B*: Western blot analysis for nitrotyrosine (*A*) in the renal cortex of the studied groups was followed by quantification of the nitrotyrosine/β-actin ratio (*B*). Values are means ± SE of 3 independent experiments for 6 rats/group. *$P = 0.01$ vs. SHR CT. Also shown are photomicrographs (*C*) and quantification (*D*) of the immunohistochemistry of oxidative stress-induced DNA damage (8-hydroxy-2-deoxyguanosine; 8-OHdG) in the kidney cortex of all studied groups. *C*: original magnification ×400 counterstained with hematoxylin. Scale bar = 50 μm. Values are means ± SE of 3 independent experiments; $n = 6$ rats/group. *$P < 0.001$ vs. SHR CT. *E* and *F*: Western blot analysis for phosphorylated AMPK (Thr[172]; pAMPK; *E*) in the renal cortex of the studied groups followed by quantification of pAMPK/TAMPK/β-actin (*F*). Values are means ± SE of 3 independent experiments; $n = 6$ rats/group. *$P < 0.0001$ vs. SHR CT.

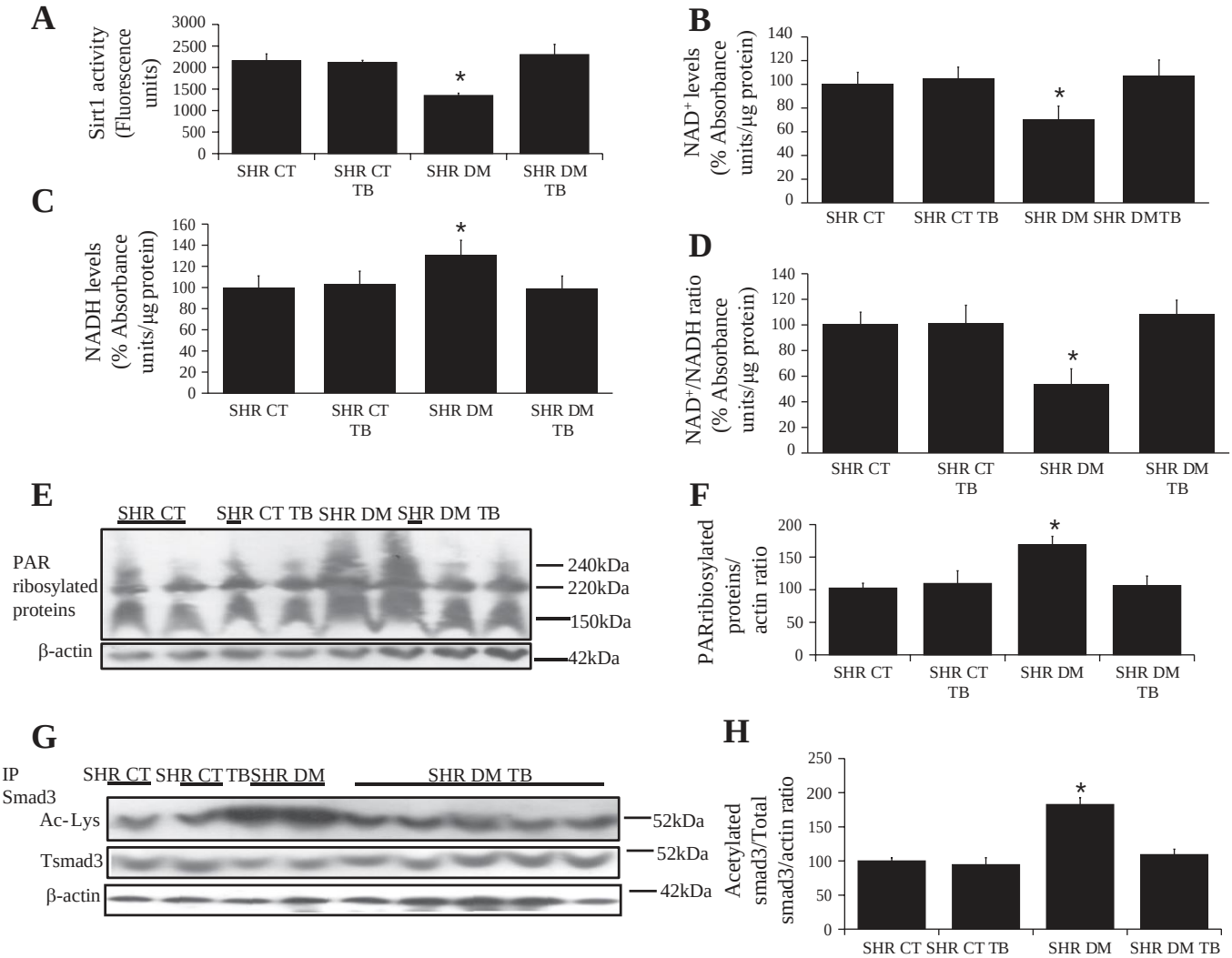
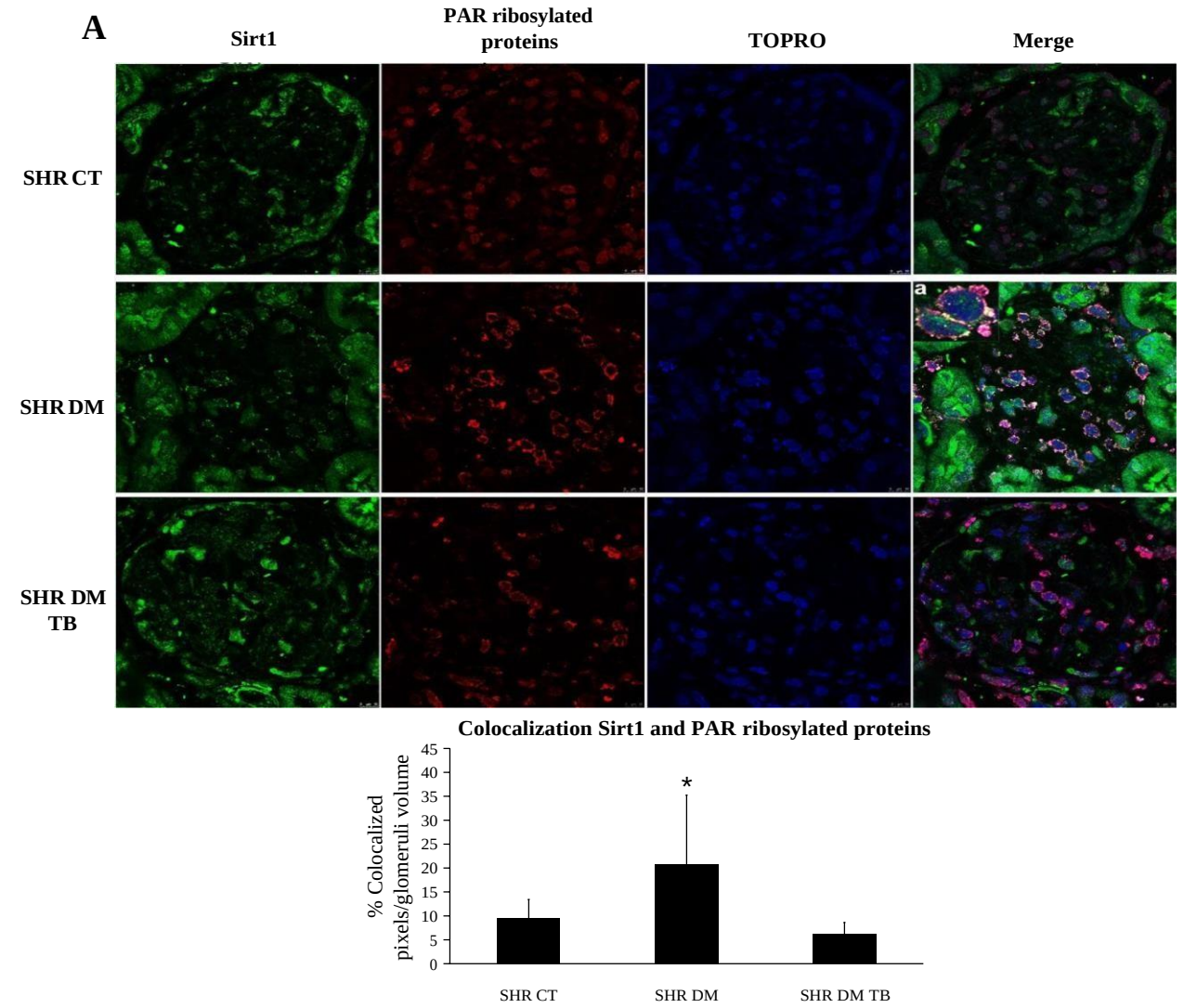


Fig. 4. Theobromine prevented reduction in Sirt-1, NAD^+/NADH ratio, and abrogated the increase in poly (ADP-ribose) polymerase (PARP) ribosylation and acetylated SMAD3 in diabetic SHR. *A*: sirtuin 1 (Sirt-1) activity levels in the studied groups. *$P < 0.0001$ vs. SHR CT. *B–D*: NAD^+ (*B*) and NADH (*C*) levels and NAD^+/NADH ratio (*D*) in the renal cortex of the studied groups. *$P < 0.005$ vs. SHR CT. Values are means ± SE of 3 independent experiments with 6 rats/group. *E* and *F*: Western blot analysis for PARP-1-induced ribosylated protein expression (*E*) in the renal cortex of the indicated groups followed by quantification (*F*) of PAR ribosylated protein/þ-actin ratio. *$P = 0.012$ vs. SHR CT. *G* and *H*: Western blot analysis of acetylated SMAD3 in renal cortex of the studied groups after immunoprecipitation of total SMAD3 and immunoblotting for acetylated SMAD3 (*G*). Western blot analysis was followed by quantification (*H*) of acetylated SMAD3/total SMAD3/þ-actin ratio. þ-Actin was used as a control of protein loading. Values are means ± SE of 3 independent experiments with 6 rats/group. *$P < 0.0001$ vs. SHR CT.

moiety. The peptide was deacetylated by Sirt-1, followed by the addition of a proteolytic developer that released the fluorescent aminomethylcoumarin. Briefly, kidney cortex extract or iHMC lysates were harvested in a RIPA buffer that was composed of 50 mM Tris·HCl (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and a protease inhibitor cocktail (Complete; Boehringer-Mannheim, Indianapolis, IN). iHMC lysates or kidney cortex extracts were collected after sonication and subsequent centrifugation at 12,000 *g* for 15 min at 4°C. A 25-µl enzyme preparation consisting of 10 µl of assay buffer provided in the kit plus 5 µl of Sirt-1 enzyme (0.2 U/µl) and 10 µl of iHMC lysate or kidney cortex extract was incubated with 25 µl containing 170 µM NAD^+ and 100 µM p53 fluorogenic peptide for 45 min at 37°C followed by incubation in a developer for 15 min at 37°C. Relative fluorescence of the fluorophore generated was measured using a fluorescence plate reader (SynergyMx; Biotek) at excitation and emission wavelengths of 360 and 460 nm, respectively. The relative fluorescence values were corrected by the amount of protein in the cell lysate or kidney cortex extract.

Fig. 5. Theobromine prevented the rise in the interaction of Sirt-1 with NOX4 and PAR ribosylated proteins and the reduction in the interaction of Sirt-1 with pAMPK (Thr^{172}). *A*: immunofluorescence staining for Sirt-1 and PAR ribosylated proteins within the glomeruli identified as green and red, respectively. Double costaining for Sirt-1 and PAR ribosylated proteins is shown as yellow. TOPRO marker shows nuclear staining. Images were analyzed by Confocal Microscopy, ×113 magnification. The quantitative analysis was performed using Image J software. Values are means ± SE of 2 independent experiments; $n = 3$ for each treatment. *B–E*: Western blot analysis of NOX4 (*B*) and pAMPK (*D*) expressions in renal cortex of the studied groups after immunoprecipitation of Sirt-1 and immunoblotting for NOX4 or pAMPK (Thr^{172}). Western blot analysis was followed by quantification of NOX4/Sirt-1/þ-actin (*C*) and pAMPK/Sirt-1/þ-actin (*E*) ratios. þ-Actin was used as a control of protein loading. Values are means ± SE of 3 independent experiments with 5 rats/group. *$P < 0.001$ vs. SHR CT.



NAD⁺, NADH, and NAD⁺/NADH ratio quantification. NAD⁺, NADH, and NAD⁺/NADH ratio were quantified using an NAD⁺/NADH quantification colorimetric kit (BioVision, Milpitas, CA) (29).

Measurement of AMP and ATP levels. The measurement of intracellular of AMP and ATP nucleotides was performed as previously described (21), with some modification. Samples of iHMCs were deproteinized in 10% TCA, mixed vigorously, and left on ice for 10 min. Homogenates were subsequently centrifuged (3,500 *g* for 10 min at 4°C), and a known volume of supernatant was collected for neutralization with a solution of 6 M KOH and 2 M K_2CO_3. The neutralized samples were centrifuged for 10 min at 3,500 *g*, and the resulting supernatants were analyzed. Protein content was determined in the TCA precipitated by using a Bradford assay. The chromatographic analysis was performed HPLC using an ACQUITY UPLC Photodiode Array (PDA, Waters). The analytic column (100 × 4·6-mm ID) was packed with 5 μm of ODS Hypersil (Thermo Scientific). Chromatographic conditions were as follows: *buffer A* consisted of 150 mM KH_2PO_4, containing 150 mM KCl, adjusted to pH 6.0 with KOH. *Buffer B* consisted of a 15% (vol/vol) solution of acetonitrile in *buffer A*. The composition of the mobile phase was controlled by a low-pressure gradient mixing device with a flow rate of 0·9 $ml^{-1} \cdot min^{-1}$ at 20°C. The gradient curve was as follows: *buffer B* was increased linearly between the following time points: 0 min, 0% B; 3·5 min, 7% B; 5·5 min, 50% B, 7·0 min, 100% B; 7·1 min, 0% B with a reequilibration time of 5 min. Sample peaks were integrated and quantified using a Waters chromatography data system. Peaks were monitored by absorption at 254 nm. A second UV detector set at 216 nm was connected in series to monitor peak purity.

Immunofluorescence for collagen type IV. Immunofluorescence staining was performed by incubating the fixed cells with collagen IV primary antibody (1:15; Southern Biotech) and rhodamine-conjugated secondary antibody for collagen IV (1:50; BD Transduction Labs, San Jose, CA). Images were then taken with an Olympus FluoView confocal microscope (Olympus of the Americas, Melville, NY).

Statistical analysis. The results, expressed as means ± SE, except for albuminuria, which is expressed as geometric mean and confidence of interval, were analyzed with the Kruskal-Wallis test (for multiple groups) and Mann-Whitney *U*-test (for 2 groups). Comparisons between multiple groups were performed with a one-way analysis of variance (ANOVA) followed by the Bonferroni test, and *t*-tests were used for comparisons between two groups. A value of $P <$

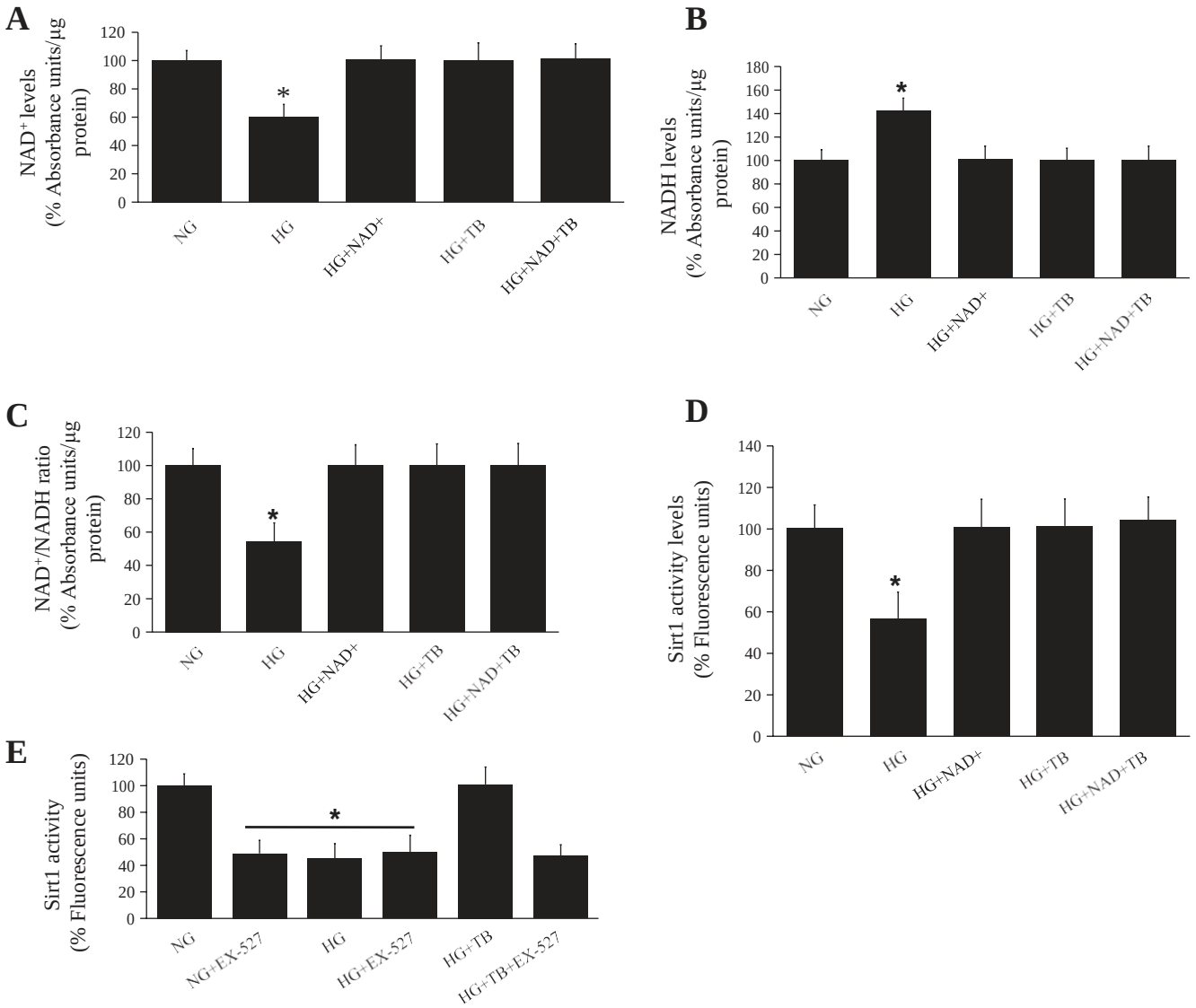


Fig. 6. Reduction of Sirt-1 in immortalized human mesangial cells (iHMCs) under high glucose (HG) is dependent on NAD⁺ depletion that is restored by theobromine. *A–E*: NAD⁺ (*A*), NADH (*B*), and NAD⁺/NADH ratio (*C*) levels and Sirt-1 activity (*D* and *E*) in iHMCs under the indicated treatments. Values are means ± SE of 3 independent experiments; $n = 6$ for each treatment. *$P <$ 0.0001 vs. normal glucose (NG).

0.05 was considered significant. The analyses were performed using StatView software (SAS Institute, Cary, NC).

RESULTS

Theobromine-attenuated diabetes-induced kidney ECM accumulation and oxidative stress in diabetic SHR. Theobromine treatment in the diabetic SHR had no effect on body weight or glycemic control, but it did reduce kidney hypertrophy (Table 2), albuminuria (Fig. 1*F*), and systolic blood pressure, although this last effect failed to reach statistical significance ($P < 0.062$). Theobromine also prevented increases in glomerular mesangial matrix expansion (Fig. 1, *G* and *H*) and collagen IV expression in the diabetic SHR (Fig. 1, *I* and *J*, and Fig. 2, *A* and *B*). In addition, theobromine treatment in diabetic SHR

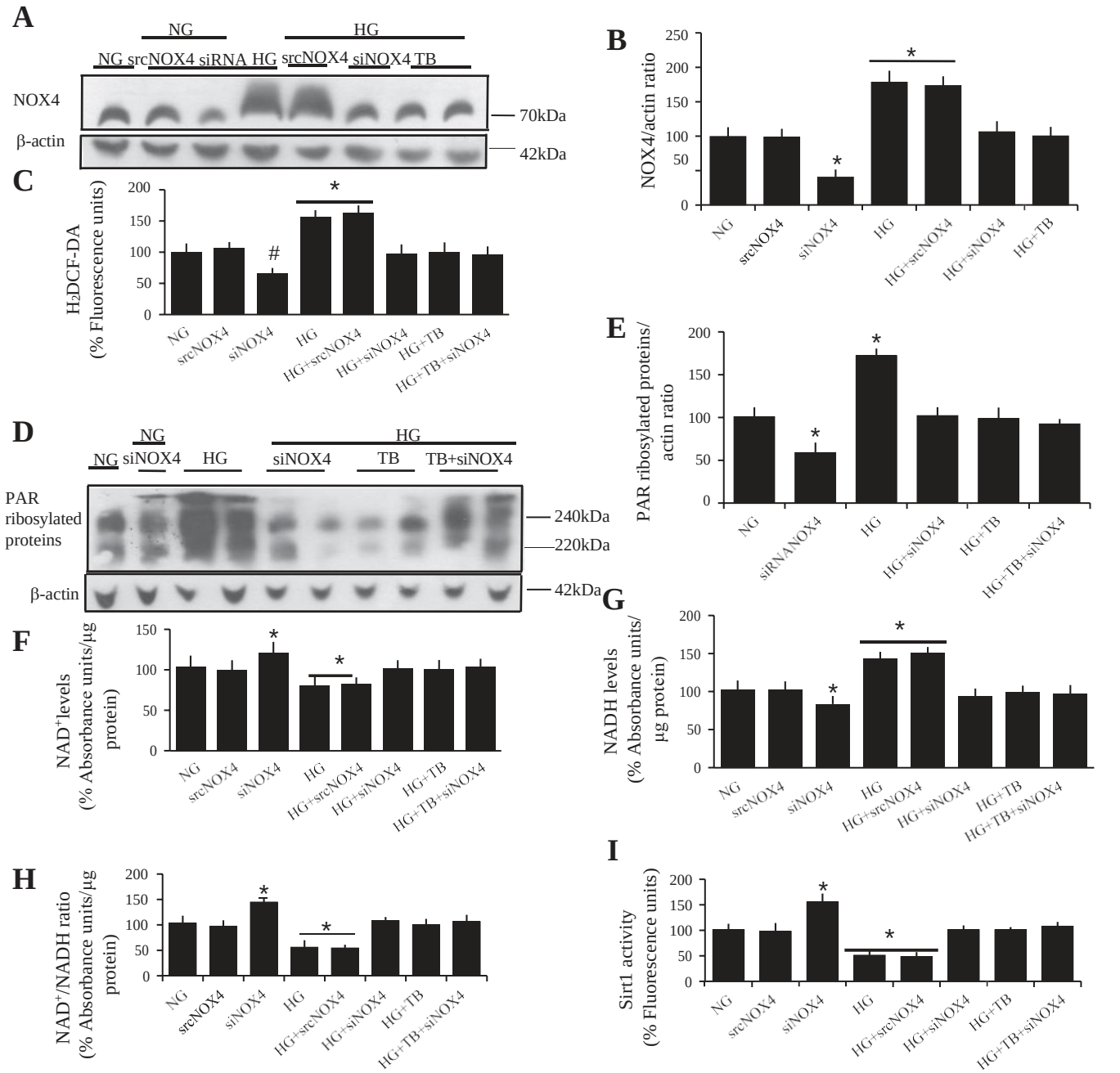


Fig. 7. In iHMCs exposed to HG, theobromine increases Sirt-1 activity via reduction in NOX4 expression and subsequent inhibition of PARP-1 activation and restoration of NAD^+ levels. *A* and *B*: Western blot analysis of NOX4 (*A*) in iHMCs treated as indicated followed by quantification of NOX4/β-actin ratio (*B*). Values are means ± SE of 3 independent experiments. $*P < 0.0001$ vs. NG. *C*: total ROS levels were quantified by incubating iHMCs with 10 µM DCF-DA for 30 min following the indicated treatments. Values are means ± SE of 3 independent experiments expressed as percentage of fluorescence units. $*P = 0.0017$ vs. NG. $\#P = 0.01$ vs. NG. *D* and *E*: Western blot analysis of PAR ribosylated protein expression (*D*) in iHMCs treated as indicated followed by densitometric analysis and quantification (*E*) of PAR ribosylated protein/β-actin ratio. Values are means ± SE of 3 independent experiments. $*P = 0.001$ vs. NG. *F–I*: NAD^+ (*F*), NADH levels (*G*), NAD^+/NADH ratio (*H*), and Sirt-1 activity (*I*) levels in iHMCs treated as indicated. Values are means ± SE of 3 independent experiments.$*P < 0.0001$ vs. NG; $n = 6$ for each treatment.

was associated with a reduction in oxidative stress parameters in the kidney. It reduced the expression of NADPH oxidase subunit NOX4 (Fig. 2, *C* and *D*) and ROS formation via NADPH oxidase (Fig. 2*E*) and ameliorated nitrosative stress- and oxidative stress-induced DNA damage (Fig. 3, *A–D*). Reduction in NADPH oxidase activity by theobromine might be mediated by activation in AMPK, as theobromine prevented diabetes-induced reduction in phosphorylated AMPK (Thr^{172}) in the kidney of SHR (Fig. 3, *E* and *F*).

Theobromine prevented reduction of Sirt-1 and acetylation of SMAD3 in the kidney of diabetic SHR. Diabetic SHR displayed a decrease in kidney Sirt-1 activity and NAD^+ levels, which are associated with increased in PARP-1 and acetylation of SMAD3 (Fig. 4, *A–H*). All these abnormalities were prevented by treatment with theobromine (Fig. 4, *A–H*).

Theobromine prevented changes in the interaction of Sirt-1 with PARP-1, Sirt-1 with p-AMPK, and Sirt-1 and NOX4 in the kidney of diabetic SHR. Diabetic SHR displayed an increase in the interaction of Sirt-1 with PARP-1 assessed by double immunofluorescent staining (Fig. 5*A*) and Sirt-1 with NOX4 (Fig. 5, *B* and *C*) and a decrease in the interaction of Sirt-1 with phosphorylated AMPK (Thr^{172}) (Fig. 5, *D* and *E*), by immunoprecipitation. All these abnormalities were prevented by treatment with theobromine (Fig. 5, *A–E*). To dissect the mechanism of Sirt-1 reduction in diabetes and its role in mediating the alleviation of ECM accumulation by theobromine under diabetic conditions, and to translate our findings from rats to a human context, we performed in vitro studies using iHMCs.

In iHMCs, HG reduces Sirt-1 activity associated with depletion of NAD^+; these abnormalities were prevented by theobromine. iHMCs exposed to HG displayed a reduction in Sirt-1 activity and NAD^+ levels that were prevented by treatment with theobromine or supplementation with NAD^+ (Fig. 6, *A–D*). A Sirt-1 activity blocker (EX-527) reversed the increasing effects of theobromine on Sirt-1 activity under HG conditions (Fig. 6*E*).

Theobromine may increase Sirt-1 activity by reducing HG-induced NOX4 expression, blocking PARP-1 activation, and restoring NAD^+ levels. Treatment with theobromine or silencing NOX4 (Fig. 7, *A* and *B*) in iHMCs under HG conditions reduced ROS formation (Fig. 7*C*) and PARP-1 activation (Fig. 7, *D* and *E*) and increased NAD^+ (Fig. 7, *F–H*) availability and Sirt-1 activity levels (Fig. 7*I*). Inter- estingly, PARP-1 blockade with a specific inhibitor, PJ-34, also increased NAD^+ levels and Sirt-1 activity in iHMCs under HG conditions (Fig. 8, *A–F*).

Theobromine prevented reduction in AMPK activity and reduced oxidative stress via inhibition of NOX4 in iHMCs exposed to HG. Quantitative (Fig. 9*A*) and qualitative (Fig. 9*B*) assessment of total ROS and ROS formation via NADPH

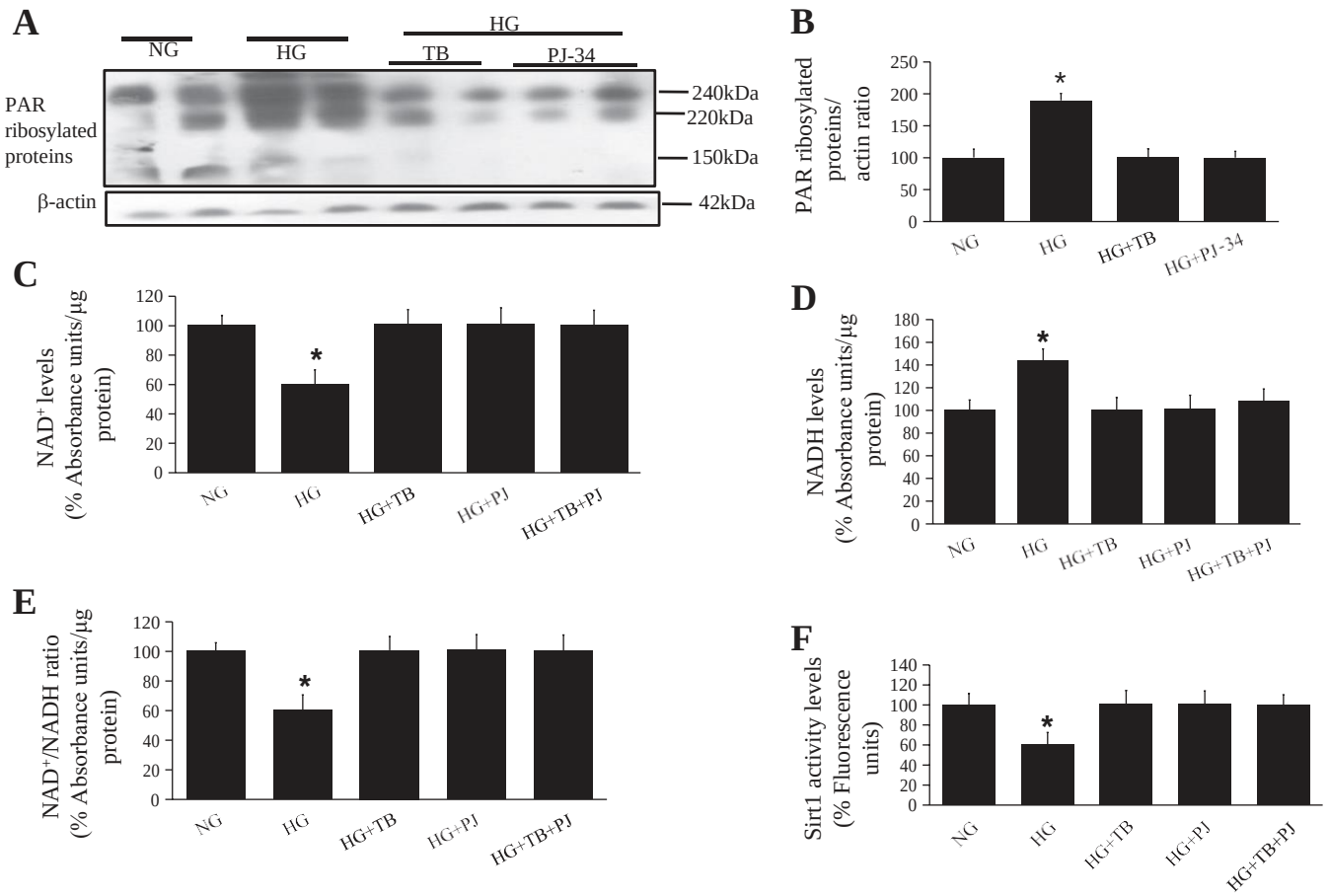


Fig. 8. In iHMCs, theobromine or PARP-1 activity blockade leads to increase in NAD^+ levels and Sirt-1 activity. Western blot analysis of PAR ribosylated protein expression in iHMCs (*A*) treated is shown followed by quantification (*B*) of PAR ribosylated protein/β-actin ratio. $*P = 0.012$ vs. NG. NAD^+ (*C*), NADH (*D*) levels, NAD^+/NADH ratio (*E*), and Sirt-1 activity levels (*F*) were assessed in iHMCs treated as indicated. Values are means ± SE of 3 independent experiments. $*P = 0.011$ vs. NG; $n = 6$ for each treatment.

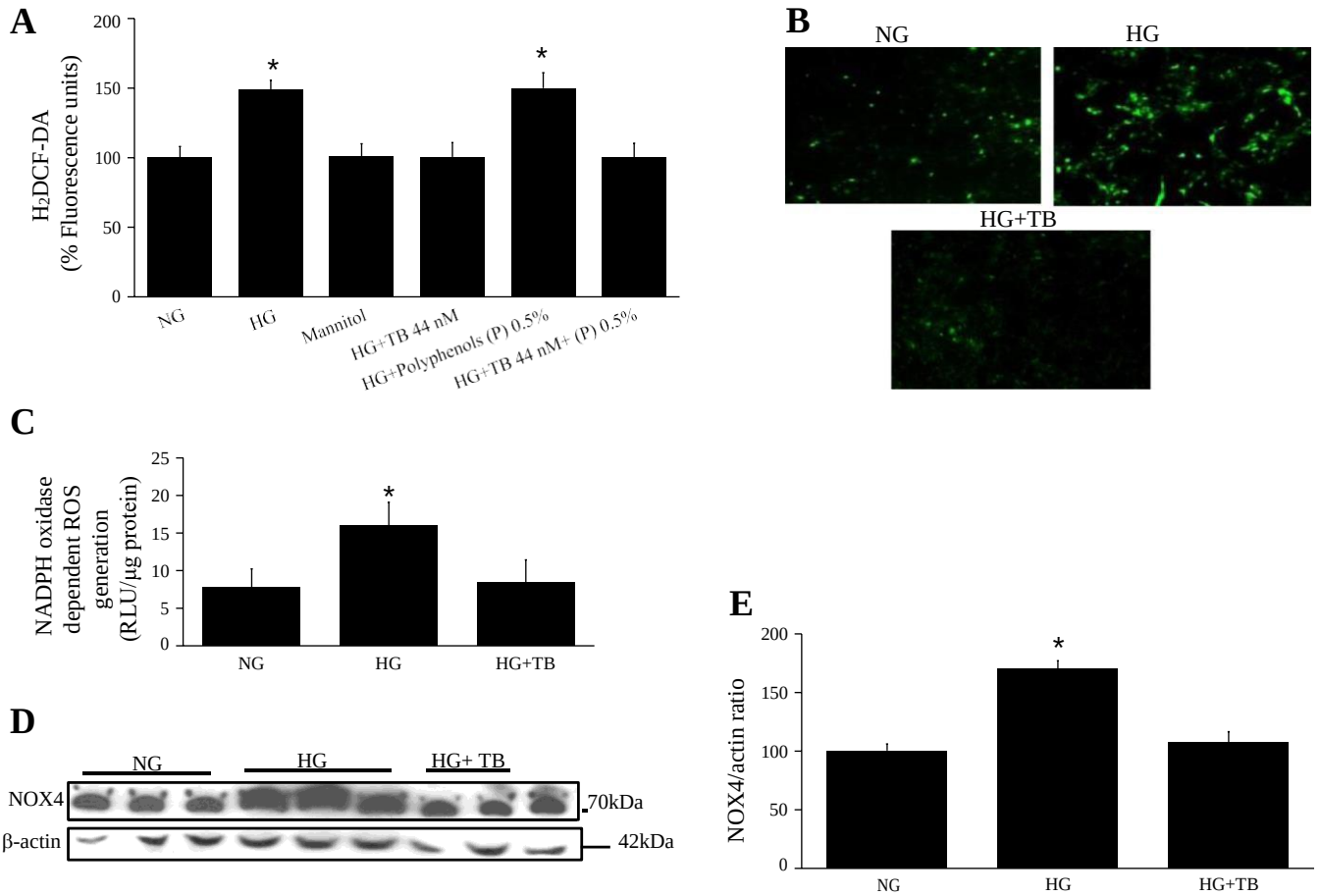


Fig. 9. Theobromine but not 0.5% polyphenols reduces ROS production via NADPH oxidase in iHMCs under HG. *A*: iHMCs in HG were treated as indicated followed by ROS quantification. Values are expressed as percentage of fluorescence units. *$P < 0.0001$ vs. NG. *B*: qualitative assessment of total ROS was carried out as in *A*. Images are illustrating ROS signal for each treatment. *C*: NADPH-dependent ROS generation in iHMCs expressed as RLU/µg protein. *$P = 0.06$ vs. NG. *D* and *E*: Western blot analysis of NOX4 in lysate of iHMCs (*D*) followed by quantification (*E*) of NOX4/þ-actin ratio. Values are means ± SE of 3 independent experiments. *$P < 0.0001$ vs. NG; $n = 6$ for each treatment.

oxidase (Fig. 9*C*) as well as NOX4 expression (Fig. 9, *D* and *E*) were significantly raised in iHMCs exposed to HG, which was prevented by theobromine. Exogenous administration of 0.5% polyphenols (Fig. 9*A*) contained within the CL did not reduce HG-induced ROS formation. Theobromine possibly blocked HG-induced NADPH oxidase activity and NOX4 expression by increasing AMPK phosphorylation (Fig. 10, *A* and *B*) via abrogating HG-induced reduction in AMP and AMP/ATP ratio levels (Fig. 10, *C–I*) since AMPK blockade prevented the effect of theobromine on NADPH oxidase activity (Fig. 11*A*) and NOX4 expression levels (Fig. 11, *B* and *C*).

Theobromine abrogated ECM accumulation via Sirt-1-induced deacetylation of SMAD3 in iHMCs under HG. Acetylated SMAD3 and collagen IV levels were significantly elevated in iHMCs exposed to HG (Fig. 12, *A–E*). These alterations induced by HG were reversed by theobromine via Sirt-1, since blockade of Sirt-1 abrogated these effects (Fig. 12, *A–E*). iHMCs exposed to TGF-þ1 also increased acetylation of SMAD3 (Fig. 13, *A* and *B*) and fibronectin expression (Fig. 13, *C* and *D*). These effects were abrogated by theobromine via Sirt-1, as silencing or pharmacological blockade of Sirt-1 impeded the protective effects of theobromine (Fig. 13, *A–D*, and Fig. 14, *A–G*).

DISCUSSION

Thus far, the mechanism of Sirt-1 depletion in the diabetic kidney has not been assessed in great detail (27, 34). Here, we showed that under diabetic conditions, Sirt-1 is reduced as a consequence of AMPK downregulation followed by NOX4 and PARP-1 activation, with subsequent NAD^+ depletion in the kidney. Following Sirt-1 reduction, kidney ECM accumulation increased due to SMAD3 acetylation induced by TGF-þ1. We also showed for the first time that a single-dose level of theobromine led to inhibition of NOX4-induced PARP-1 activation, possibly by preventing AMPK inactivation via an increase in AMP/ATP ratio levels (15, 17). This effect led to an increase in NAD^+ levels, and hence, Sirt-1 activation, which prevented SMAD3 acetylation protecting the diabetic kidney from ECM accumulation (Fig. 15). Of interest, we have recently demonstrated (43), by matrix-free nano-assisted laser desorption-ionization mass spectrometry imaging, that indeed theobromine accumulates in the kidney after oral administration of theobromine to rats (Drug Testing). This latter finding further supports the concept that theobromine may have a potential as a renal-protective drug.

ECM accumulation is a hallmark of kidney disease in DM (49). Some studies have shown that in DM reduction in the

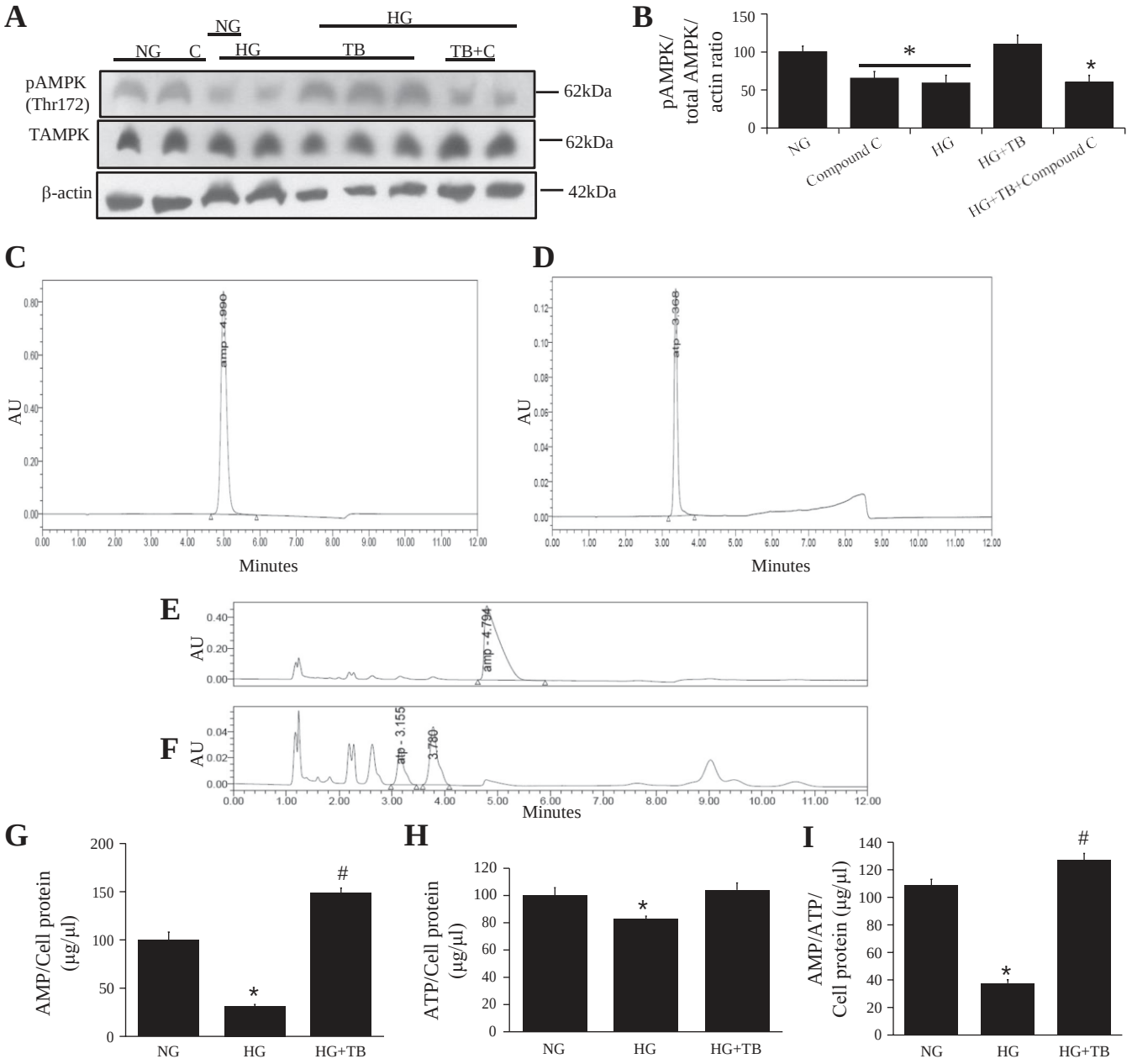


Fig. 10. Theobromine treatment in iHMCs abrogates HG-induced AMPK inactivation via rise in AMP/ATP ratio levels. *A* and *B*: Western blot analysis of phosphorylated AMPK (Thr^{172}) in iHMCs (*A*) treated as indicated followed by quantification of pAMPK/TAMPK/β-actin ratio (*B*). Values are means ± SE of 3 independent experiments. *$P < 0.0001$ vs. NG. *C* and *D*: chromatography analysis of AMP (*C*) and ATP (*D*) standards and their corresponding retention times. Peaks represent AMP (retention time 5.02) and ATP (retention time 3.34). *E* and *F*: representative chromatography analysis of AMP (*E*) and ATP (*F*) in iHMCs treated with NG for 24 h. *G–I*: AMP (*G*), ATP (*H*), and AMP/ATP ratio (*I*) levels were measured by HPLC and corrected for protein content in iHMCs treated as indicated. Values are means ± SE of 3 independent experiments; $n = 5$ for each treatment. *$P < 0.0001$ vs. NG (*G*). *$P = 0.02$ vs. NG (*H*). *$P < 0.0001$, #$P < 0.0001$ vs. NG (*I*).

kidney expression and activity of Sirt-1 are associated with ECM accumulation and an elevated expression of TGF-β1 (30, 41). However, these studies did not assess the mechanism by which Sirt-1 reduction leads to ECM accumulation. A possible explanation is the reduction of Sirt-1 activity on deacetylating SMAD2 and SMAD3 (31). Acetylation involves the transfer of the acetyl moiety from acetyl coenzyme A to the ε-amino groups of lysine residues (31, 48). SMAD3 can be acetylated at the Lys-378 in the MH2 domain by p300/CBP (31, 20), which regulates SMAD3 DNA binding activity and subsequently mediates TGF-β1-induced collagen synthesis in fibroblasts (3), tubular cells (31), and 293 and HepG2 cell lines (3), suggesting that the acetylation of SMAD3 might play an important role in TGF-β1-driven tissue fibrosis. Our results support these studies, showing that acetylation in SMAD3 is vital in mediating diabetes or HG-induced renal ECM accumulation, possibly due to the lack of deacetylating activity of Sirt-1. This is the first description of a Sirt-1/SMAD3 interaction in DN and may represent a common mechanism in the regulation of members of the SMAD family, as a previous study reported that Sirt-1

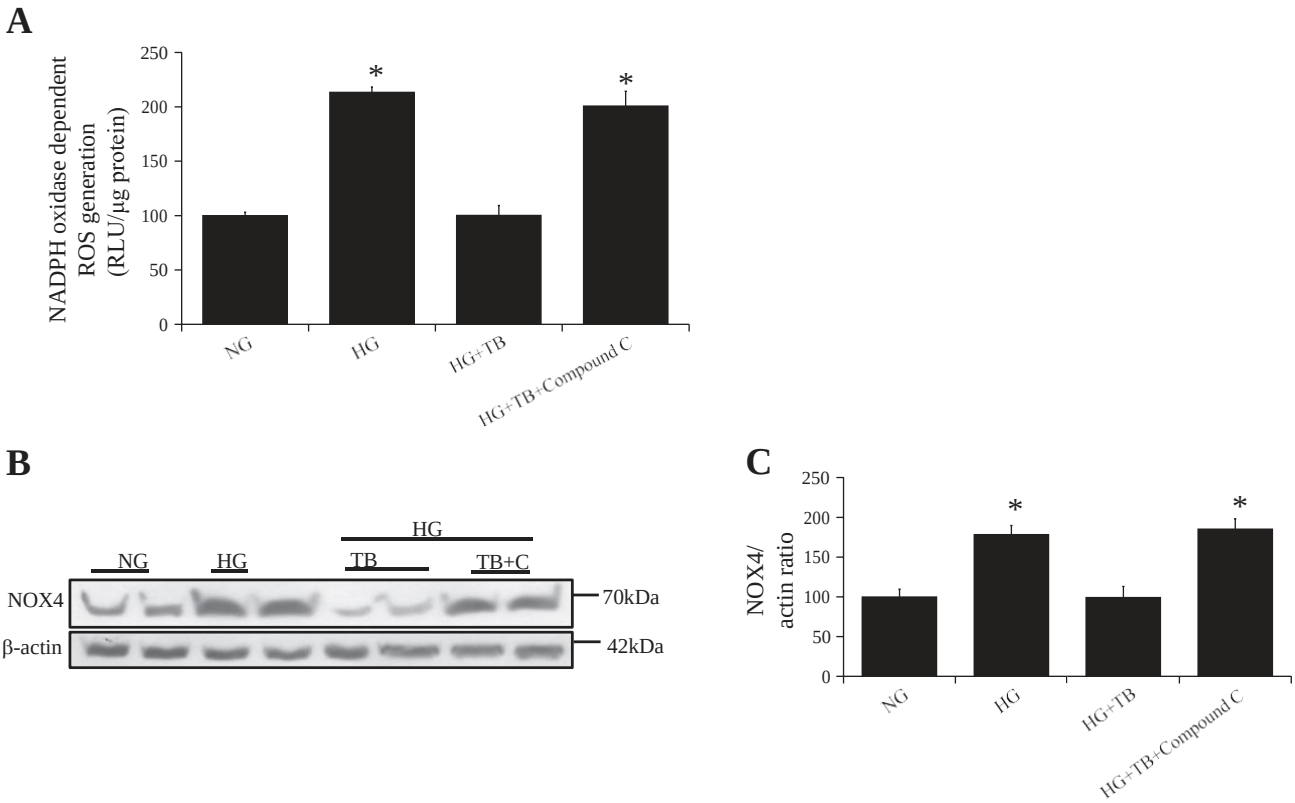


Fig. 11. Theobromine treatment in iHMCs exposed to HG reduces NOX4 expression via AMPK activation. *A*: NADPH-dependent ROS generation in iHMCs treated as indicated and expressed as RLU/μg protein. Values are means ± SE of 3 independent experiments. **P* = 0.0011 vs. NG. *B* and *C*: Western blot analysis of NOX4 in iHMCs treated as indicated (*D*) followed by densitometric analysis and quantification of NOX4/β-actin ratio (*E*). Values are means ± SE of 3 independent experiments. **P* = 0.0025 vs. NG; *n* = 6 for each treatment.

can deacetylate SMAD7, which prevents TGF-β1-induced apoptosis in cultured glomerular mesangial cells (28).

Dietary restriction (29, 26, 44) and maneuvers via the use of plant-derived polyphenols such as resveratrol (24, 45–47) have been shown to ameliorate renal injury in experimental diabetes via an association with the regulation of Sirt-1 protein. In particular, CR or activation of Sirt-1, has been associated with alleviation of ECM accumulation in experimental type 2 diabetes (26) and a unilateral ureteral obstruction model (31). In vitro, Sirt-1 activation or overexpression has been shown to ameliorate ECM accumulation in both mesangial cells exposed to HG (30, 41) and tubular and fibroblast kidney cells treated with TGF-β1 (31). A few years ago, it was suggested that theobromine, a methylxanthine present in cocoa, could at least partly account for the salutary effects of cocoa in humans (23). More recently, a number of potential beneficial actions of theobromine have been described, including a capacity to enhance HDL cholesterol concentrations in healthy volunteers (35). Of interest theophylline, a methylxanthine derivative (a component of tea polyphenol), protects against intracellular NAD^+ depletion via PARP-1 inhibition and thus activates Sirt-1 activity in the macrophages and lung cells of patients with chronic obstructive pulmonary disease (34).

Interestingly, while no study has been conducted on the effect of theobromine on diabetic nephropathy, a PubMed search with the terms "theobromine and diabetic nephropathy" resulted in 51 articles, most of which were related to the use of pentoxifylline in diabetic nephropathy. The reason might be that pentoxifylline, like theobromine, is a xanthine derivative. Pentoxifylline is a commercially available drug that is mostly indicated in the treatment of intermittent claudication. To date, a few studies have demonstrated a reduction in albuminuria in diabetic patients treated with pentoxifylline, with almost no side effects, although its real contribution in treating DN needs to be tested in large, randomized clinical trials (40).

In the current study, we observed a nonsignificant reduction in blood pressure by theobromine in diabetic rats, which might have contributed to the beneficial effects of theobromine. In addition, the theobromine treatment in the diabetic rats was initiated soon after the induction of diabetes and before the development of renal disease, which is a situation that is not likely to occur clinically. Finally, we did not assess whether the effect of theobromine on phosphodiesterase inhibition, interfering with adenosine 3′,5′-phosphate cAMP signaling, contributed to the observed protective effects of theobromine. However, it is generally accepted that theobromine is a weaker phosphodiesterase inhibitor compared with other methylxanthines such as caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) (32, 39).

In conclusion, in this study we identified a novel regulatory mechanism for Sirt-1 reduction in the diabetic kidney that involves the PARP-1 activation effect of increased ROS production by NADPH-enhanced NOX4 via AMPK inactivation, leading to the depletion of NAD^+. The consequent reduction in Sirt-1 activity impairs SMAD3 deacetylation with the development of kidney ECM accumulation. AMPK activation by

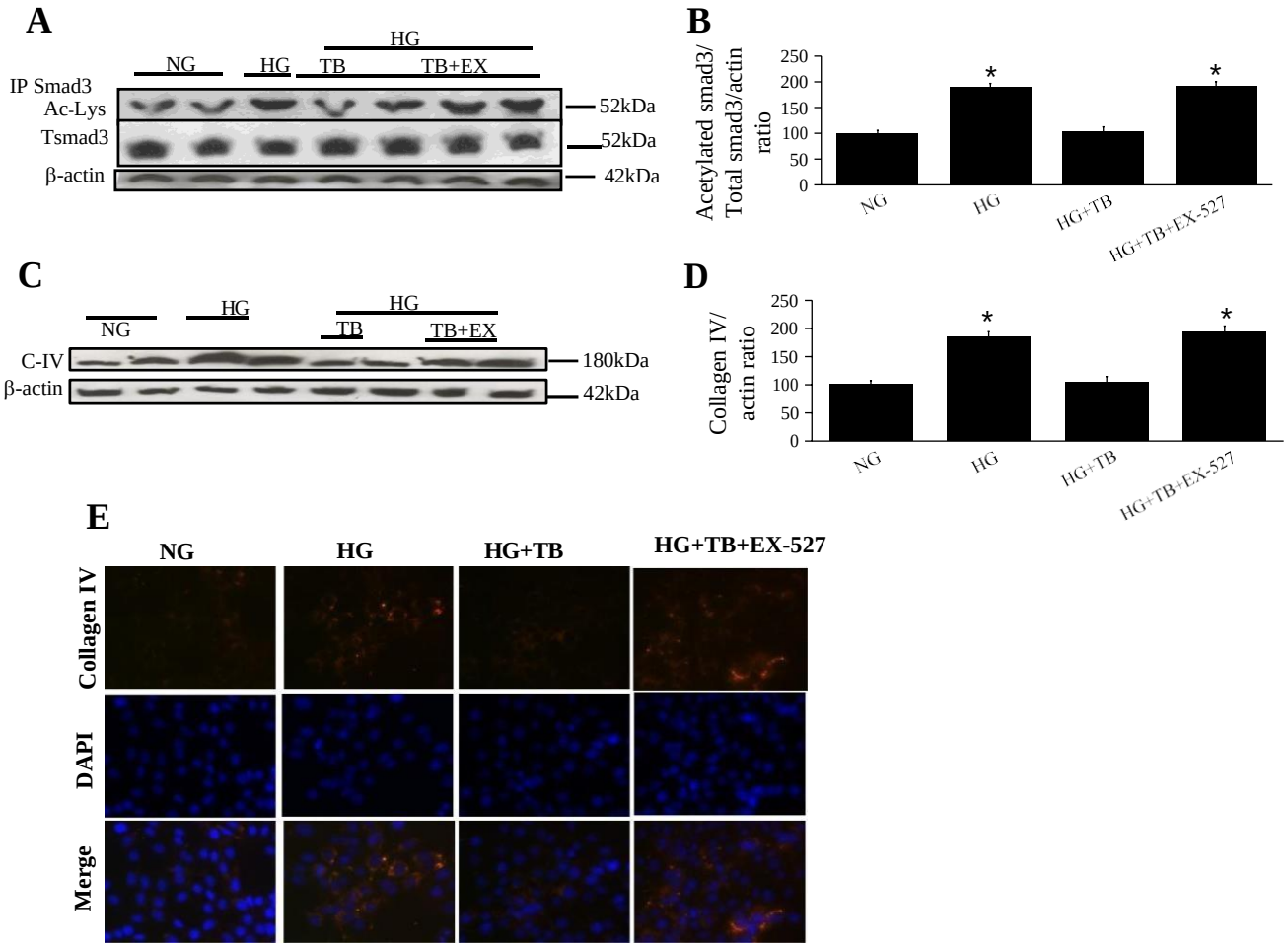


Fig. 12. Theobromine in iHMCs reduces HG-induced ECM accumulation via Sirt-1-induced SMAD3 deacetylation. Shown are Western blot analysis of acetylated SMAD3 (*A*) and collagen IV (*C*) in iHMCs treated as indicated and quantification of acetylated SMAD/total SMAD3/þ-actin (*B*) and collagen IV/þ-actin (*D*) ratios. Values are means ± SE of 3 independent experiments. *$P < 0.001$ vs. NG; $n = 6$ for each treatment. *E*: immunofluorescence staining for collagen IV in iHMCs treated as indicated.

theobromine is able to interrupt the sequence of events leading to Sirt-1 activation, and hence, protects the diabetic kidney.

ACKNOWLEDGMENTS

The authors thank Luciana Cristina Teixeira (Department of Medicine, Nephrology Division, Federal University of São Paulo, São Paulo, Brazil) for helping with the culture of the iHMC line and are grateful to Rita de Cassia Zacardi de Souza, Dr. Ricardo Pereira, and Dr. Marcelo Ganzarolli de Oliveira [Laboratory of Liquid Chromatography, Institute of Chemistry, University of Campinas (Unicamp), Campinas, São Paulo, Brazil] for technical and scientific assistance. The authors are grateful to Felipe Franco da Rocha and Janine Sabino (Central Laboratory of High Performance Technologies in Life Sciences-LACTAD, University of Campinas, Campinas, São Paulo, Brazil) for technical and scientific assistance in immunofluorescences analysis. The authors are very grateful to the personnel from the Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, Faculty of Medical Sciences (FCM), Unicamp, for invaluable help with this work, in particular Diego A. Duarte and Virginia de Campos Carvalho.

GRANTS

This work was supported by the Fundação de Amparo a`Pesquisa do Estado de São Paulo (FAPESP; Grant 2008/57560-0 and 2012/22452-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grant 301797/2009-9). A. Papadimitriou received a postdoctoral scholarship from FAPESP.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.P., K.C.S., E.B.P., J.M.L.F., and J.B.L.F. provided conception and design of research; A.P., K.C.S., E.B.P., C.M.B. performed experiments; A.P., K.C.S., E.B.P., J.M.L.F., and J.B.L.F. analyzed data; A.P., K.C.S., E.B.P., J.M.L.F., and J.B.L.F. interpreted results of experiments; A.P., K.C.S., E.B.P. prepared figures; A.P., K.C.S., E.B.P. drafted manuscript; A.P., K.C.S., E.B.P., J.M.L.F., and J.B.L.F. edited and revised manuscript; A.P., K.C.S., E.B.P., C.M.B., J.M.L.F., and J.B.L.F. approved final version of manuscript.

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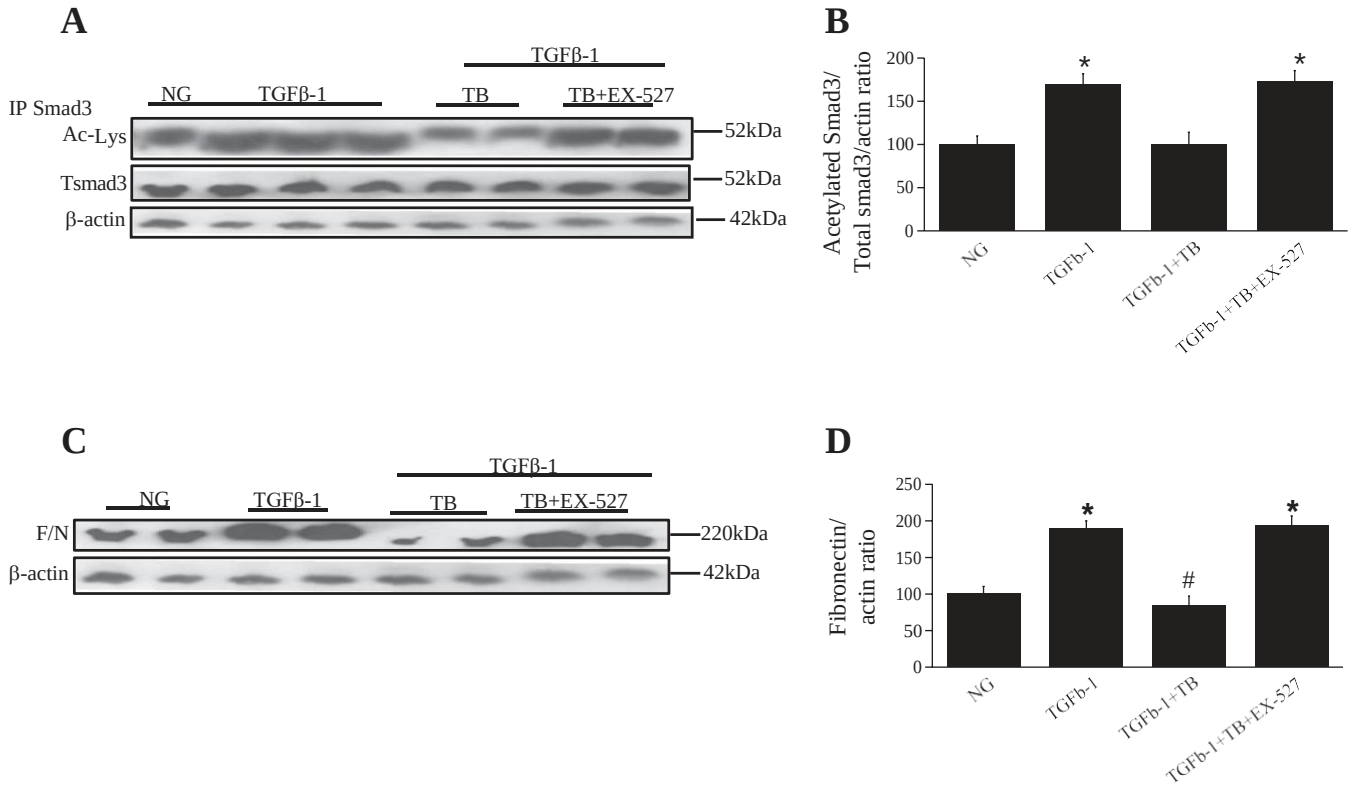

Fig. 13. In iHMCs exposed to TGF-þ1, theobromine decreases ECM accumulation by deacetylation of SMAD3. Shown are Western blot analysis of acetylated Smad3 (*A*) and fibronectin (*C*) in iHMCs treated as indicated and quantification of acetylated Smad3/total Smad3/þ-actin (*B*) and fibronectin (F/N)/þ-actin (*D*) ratios. Values are means ± SE of 3 independent experiments; $n = 6$ for each treatment. *$P = 0.003$ vs. NG (*B*). *$P < 0.0001$, #$P = 0.05$ vs. NG (*D*).

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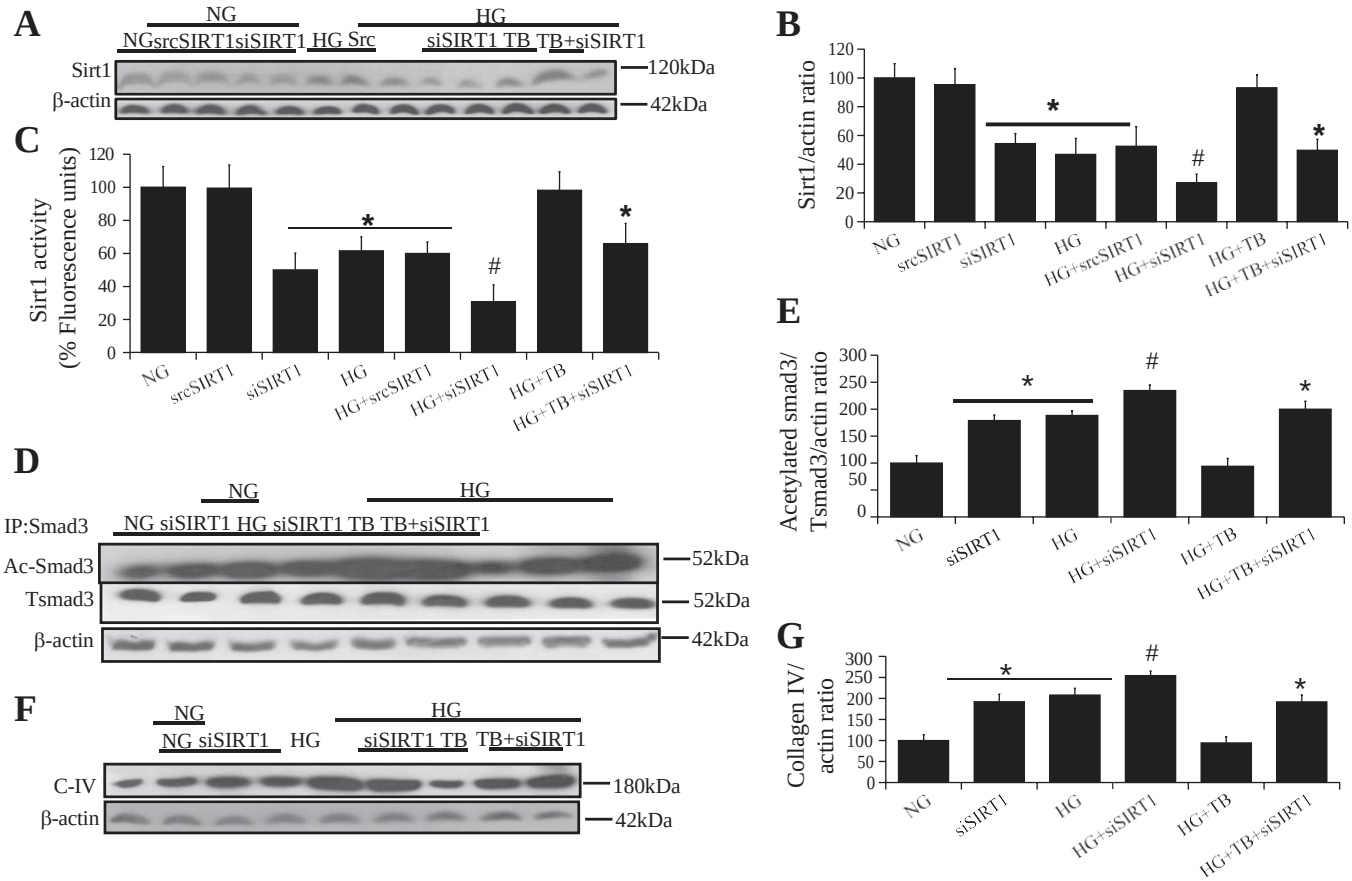

Fig. 14. Silencing Sirt-1 prevents protective effect of theobromine treatment in iHMCs exposed to HG against collagen IV accumulation via increase in acetylation of SMAD3. *A* and *B*: Western blot analysis of Sirt-1 (*A*) in iHMCs treated as indicated followed by quantification of Sirt-1/þ-actin ratio (*B*). Values are means ± SE of 3 independent experiments. *$P < 0.0001$ vs. NG. #$P = 0.01$ vs. HG *C*: Sirt-1 activity levels in iHMCs treated as indicated. Values are means ± SE of 3 independent experiments. *$P = 0.002$ vs. NG. #$P = 0.01$ vs. HG. *D–G*. Western blot analysis of acetylated SMAD3 (*D*) and collagen IV (*F*) in iHMCs treated as indicated. Also shown is quantification of acetylated SMAD3/total SMAD3/þ-actin (*E*) and collagen IV/þ-actin (*G*) ratios. Values are means ± SE of 3 independent experiments; $n = 4$ for each treatment. *$P = 0.012$ vs. NG, #$P = 0.01$ vs. HG (*E*). *$P < 0.001$ vs. NG, #$P = 0.02$ vs. HG (*G*).

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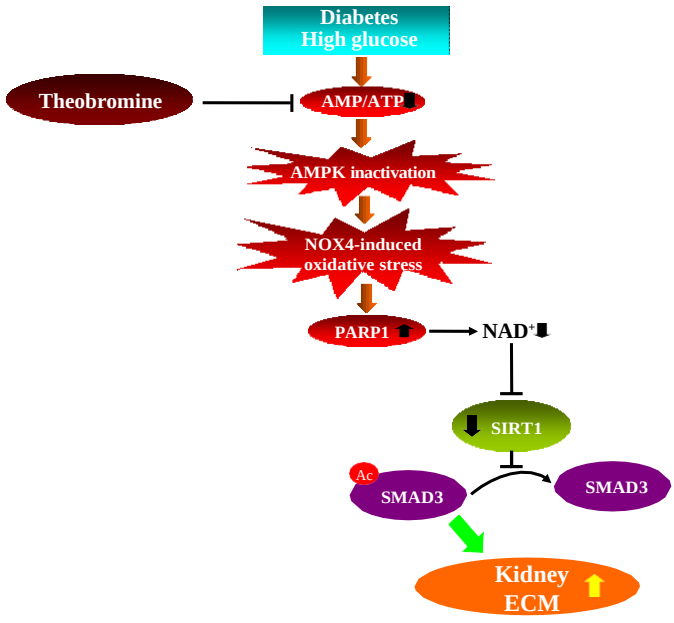

Fig. 15. Schematic representation of the proposed mechanism by which diabetes/HG leads to Sirt-1 downregulation and ECM accumulation and the inhibitory action of theobromine in this sequence of events.

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