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FLAVINAS PROMOVEM MUDANÇAS NA MATRIZ EXTRACELULAR, VIAS DE TRANSDUÇÃO DE SINAL, ENZIMAS ANTIOXIDANTES E METALOPROTEINASES DURANTE A DIFERENCIAÇÃO DE OSTEOBLASTOS

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Antonio H. Chaves Neto
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Orientadora: Profa. Dra. Carmen Veríssima Ferreira

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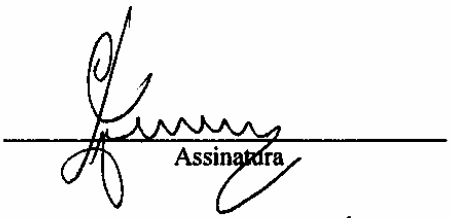
Profa. Dra. Carmen Veríssima Ferreira (Orientadora) 
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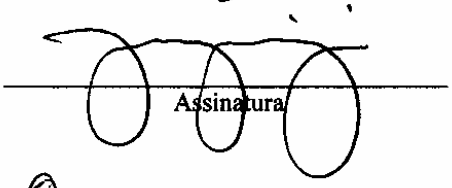
Prof. Dr. José Mauro Granjeiro 
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Epígrafe

"O destino não é uma questão de sorte, é uma questão de escolha; não é algo a se esperar, é algo a se conquistar".

(William Jennings Bryan)

Resumo

Chaves Neto AH. Flavinas promovem mudanças na matriz extracelular, vias de transdução de sinal, enzimas antioxidantes e metaloproteinases durante a diferenciação de osteoblastos. Campinas, 2009. 123p. Tese (Doutorado em Biologia Funcional e Molecular, área de concentração Bioquímica) UNICAMP – Universidade Estadual de Campinas, Instituto de Biologia.

Riboflavina (Rb – Vitamina B2) é o precursor das flavocoenzimas essenciais flavina mononucleotídeo (FMN) e flavina adenina dinucleotídeo (FAD). Estas coenzimas participam de processos enzimáticos dependentes das reações de transferências de elétrons, que ocorrem nas vias de produção de energia, biossíntese, desintoxicação e sequestro de elétrons. O aumento dietético da riboflavina e piridoxina foi associado com maiores densidades minerais em mulheres e homens idosos. Fotoderivados da riboflavina demonstraram efeitos citotóxicos em células cancerosas de próstata e leucemias, entretanto, o efeito direto da Rb e seus fotoderivados em osteoblastos não foram examinados. Neste trabalho os efeitos biológicos da Rb e riboflavina irradiada (IRb) foram investigados na linhagem de pré-osteoblastos MC3T3-E1, um modelo bem aceito de osteogênese *in vitro* caracterizado pela indução de genes específicos associados com o fenótipo osteoblástico quando tratados com ácido ascórbico e β-glicerofosfato. A viabilidade celular foi avaliada através da redução do MTT, da incorporação do corante vermelho neutro e do conteúdo de ácidos nucléicos. Marcadores de diferenciação osteoblástica foram analisados através do RT-PCR semi-quantitativo (osteopontina e osteocalcina) e através de análises colorimétricas de atividade da fosfatase alcalina (FAL) e síntese de colágeno pela coloração de picrosirius. As atividades das metaloproteinases (MMP) -9 e -2 foram avaliadas pela zimografia de gelatina. Microarranjos de peptídeos com subtratos específicos para quinases e imunoblotting foram usados para identificar os efeitos na sinalização celular. As atividades de enzimas antioxidantes (superóxido dismutase, catalase, glutationa peroxidase e glutationa S-transferase) foram determinadas em lisados celulares usando métodos espectrofotométricos. As atividades das caspases-8, -9 e -3 foram analisadas através de métodos colorimétricos. Na primeira análise Rb e IRb causaram a parada do ciclo celular na fase G_0/G_1 e também a inibição da quinase AKT, um mediador da proliferação. Flavinas causaram a diferenciação de pré-osteoblastos, evidenciada

pelo aumento da expressão de osteocalcina, osteopontina e BMP-2. Atividades mais elevadas de MMP-9 e MMP-2 também foram observadas. A capacidade das flavinas em engatilhar a diferenciação de osteoblastos foi reforçada pelo aumento da conexina 43, diminuição da caveolina-1 e repressão da sinalização Notch. Na segunda análise, nós encontramos que as interações entre Rb, em sua forma irradiada e não-irradiada, e indutores osteogênicos (ácido ascórbico e β-glicerofosfato) afetaram significativamente a proliferação de osteoblastos, a atividade de FAL, biossíntese de colágeno, expressão de osteocalcina e osteopontina, a atividade das MMP-2 e MMP-9 e a expressão de fatores osteoclastogênicos (RANKL e osteoprotegerina). Nós também encontramos que os efeitos das flavinas em osteoblastos nesta segunda etapa foram independentes das suas propriedades antioxidantes. A atividade biológica da combinação de indutores osteogênicas com Rb e seus fotoprodutos foi associada com a ativação de diferentes vias de sinalização (AKT, FAK, CaMKII), caspases -8, -9 e -3 e aumento da expressão e/ou estabilização de fatores de transcrição osteoblásticos (Runx2 e β-catenin). Este estudo nos trouxe fortes evidências que altas concentrações de Rb e IRb geraram um microambiente osteogênico através da modulação de diferentes vias de sinalização, além de promover um efeitos aditivo durante a diferenciação das células pré-osteoblasticas MC3T3 induzida por ácido ascórbico e β-glicerofosfato. Em resumo, este estudo aponta para uma potencial aplicação da Rb e seus fotoprodutos no desenvolvimento do fenótipo osteoblástico e, consequentemente, uma alternativa terapêutica coadjuvante para osteoporose.

Palavras-chave: 1. Transdução de sinal celular. 2. Riboflavina. 3. Osteoblastos. 4. Células - Diferenciação.

Abstract

Chaves Neto AH. Flavins promote changes in the extracellular matrix, signal transduction, antioxidant enzymes and metalloproteinases during osteoblast differentiation. Campinas, 2009. 123p. Tese (Doutorado em Biologia Funcional e Molecular, área de concentração Bioquímica) UNICAMP – Universidade Estadual de Campinas, Instituto de Biologia.

Riboflavin (Rb-Vitamin B2) is the precursor of essential flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes participate in numerous enzymatic processes dependent on electron transfer reactions that occur in energy-producing, biosynthetic, and detoxifying and electron-scavenging pathways. Increase dietary riboflavin and pyridoxine intake has been associated with higher bone mineral density in elderly men and women. Photoderivatives of riboflavin have been shown strong activity in haematological malignancy and prostate cancer cells, however, the direct effect of Rb and its photoderivatives on osteoblast has not been examined. In this work, the biologic effects of Rb and irradiated riboflavin (IRb) were investigated in the MC3T3-E1 pre-osteoblastic cell line, a well-accepted model of osteogenesis *in vitro* characterized for the induction of specific genes associated with the osteoblastic phenotype when treated with ascorbic acid and β-glycerophosphate. Cell viability was assessed by MTT reduction, neutral red uptake and nucleic acids content. Osteoblastic differentiation markers were analyzed by semiquantitative RT-PCR (osteopontin and osteocalcin), alkaline phosphatase (ALP) activity measured colorimetrically and collagen synthesis by Sirius red staining. Metalloproteinases (MMP) -9 and -2 activities were assayed by gelatin zymography. Peptide microarray of substrate specificity to kinases and immunoblotting were used to identify the effects on signal transduction pathways. Antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase) were determined in cellular lysate using spectrophotometric methods. Caspase-8, -9 and -3 activation were measured by a colorimetric assay. In the first analysis Rb and IRb caused cell cycle arrest at G_0/G_1 phase and accordingly inhibited AKT kinase, a proliferation mediator. Flavins caused differentiation of preosteoblast cells as evidenced by increase of osteocalcin, osteopontin and BMP2 expressions. In addition, higher MMP-9 and -2 activities were observed.

Importantly, the capacity of flavins to trigger osteoblasts differentiation was also reinforced by upregulation of connexin 43, down regulation of caveolin-1 and negative modulation of Notch cascade. In the second analysis, we found that the interaction between Rb and IRb and osteogenic inductors (ascorbic acid and β-glycerophosphate) significantly affected the osteoblast proliferation, alkaline phosphatase activity, collagen biosynthesis, osteopontin and osteocalcin mRNA expression, MMP-2 and MMP-9 activities and the expression of osteoclastogenesis factors (RANKL and OPG). We also showed that the effects of flavins in osteoblasts cells were independent on flavins antioxidant property. The biological activity of the combination of osteogenic medium with riboflavin and its photoderivatives was associated with the activation of different signaling pathways (AKT, FAK, CaMKII), caspases -8, -9 and -3, and up-regulation and/or stabilization of osteoblastic transcription factors (Runx2 and β-catenin). This study brought out strong evidences that high concentration of Rb and IRb generates an osteogenic microenvironment through modulating different mediators of signaling pathways, besides of the additive effect of riboflavin and its photoproducts during the ascorbate and β-glycerophosphate-induced osteoblast differentiation of MC3T3-E1 cells. In summary, this study pointed out the potential application of Rb and its photoproducts in osteoblasts phenotype development and, consequently, it is possible use as an alternative therapeutic adjuvant of osteoporosis.

Key-words: 1. Cellular signal transduction. 2. Riboflavin. 3. Osteoblast. 4. Cell differentiation.

Sumário

Introdução

Introdução

O esqueleto serve para uma variedade de funções. Os ossos do esqueleto proveem suporte estrutural para todo o corpo, permite movimentos e a locomoção servindo como alavancas para os músculos, protegem órgãos e estruturas vitais internas, prove a manutenção da homeostasia mineral e balança ácido-base, serve como reservatório de fatores de crescimento para a hematopoiese dentro dos espaços da medula óssea (Taichman, 2005).

1 Características normais do osso

1.1 Morfologia

Os ossos de um esqueleto adulto são compostos primariamente de dois tipos de tecido ósseo: o cortical ou compacto e osso medular ou esponjoso. A maioria dos ossos consiste de uma cortical externa que encerra uma rede trabecular de osso esponjoso, a qual abriga a medula óssea. A cortical óssea é revestida na sua parte externa e interna pelo periósteo e endósteo, respectivamente. O endósteo da cortical óssea é conectado ao osso esponjoso e consiste de placas e hastes interconectadas. Esta estrutura maximiza a força, enquanto minimiza o peso. As placas e hastes da rede de tecido ósseo esponjoso são preferencialmente orientadas ao longo de linhas de tensão mecânica do osso (Kanis, 1994; Einhorn, 1996; Fleisch, 1997).

A cortical óssea compreende 80% do esqueleto e o osso trabecular 20%, o anterior fornece aproximadamente 75% da massa óssea, enquanto o último aproximadamente 75% da superfície óssea (Deftos, 1998). Entretanto, as proporções relativas de osso cortical ou esponjoso variam em diferentes partes do esqueleto (Kanis, 1994; Einhorn, 1996; Fleisch, 1997).

1.2 Composição do osso

1.2.1 Mineriais ósseos

O componente mineral do osso corresponde a aproximadamente 65% de seu peso total seco. Quimicamente, ele é predominantemente hidroxiapatita, $Ca_{10}(PO_4)_6(OH)_2$. Outros

constituintes, tais como carbonatos, citratos, sódio, fluoretos e estrôncio, são incorporados dentro da estrutura interna do cristal de hidroxiapatita ou adsorvidos a sua superfície. Algumas substâncias, como os bisfosfonatos, têm uma especial afinidade para a porção mineral do osso (Kanis, 1994; Einhorn, 1996; Fleisch, 1997).

1.2.2 Matriz orgânica óssea

A matriz orgânica óssea corresponde a aproximadamente 35% do peso seco total do osso. Aproximadamente, 90% desta matriz consiste de proteínas colágenas específicas de tecido ósseo; o remanescente consiste de proteínas não colágenas, tais como osteonectina, osteocalcina (formalmente referida como proteína óssea Gla), osteopontina e sialoproteína óssea. As proteínas de matriz óssea são sintetizadas e secretadas pelos osteoblastos. Fibras colágenas são habitualmente orientadas em uma direção preferencial, o que leva a uma estrutura lamelar típica. As lamelas são geralmente paralelas umas às outras se depositadas ao longo de superfícies planas, tais como as superfícies da rede trabecular ou do periósteo, ou concêntricas, se sintetizadas dentro da cortical óssea em uma superfície que limita um canal, o qual centraliza um vaso sanguineo. Estas estuturas concêntricas dentro da cortical óssea são conhecidas como ósteon ou sistema Haversiano (Robey & Boskey, 1996; Eyre, 1996). A concentração plasmástica e/ou urinária da excreção de produtos colágenos e certas proteínas não colágenas, como osteocalcina, reflete a taxa de formação e de reabsorção óssea (Garnero & Delmas, 1998) e são usadas clinicamente como marcadores bioquímicos de turnover ósseo.

1.2.3 Células ósseas

Osteoblastos são células formadoras de osso. Elas são originadas a partir de células-tronco mesenquimais (estroma da medula óssea ou mesênquima do tecido conjuntivo), as quais sofrem proliferação, diferenciação em pré-osteoblastos e na sequencia tornam-se osteoblastos maduros (Triffitt, 1996). Os osteoblastos formam uma estrutura de revestimento unidirecional nas

superfícies da matriz orgânica. A espessura desta camada, chamada de osteóide, depende simultaneamente da formação da matriz orgânica e sua subseqüente calcificação – processo denominado primariamente de mineralização. Sistemas de transportes, localizados na membrana plasmática dos osteoblastos, são responsáveis por transferirem íons do osso mineralizado, principalmente cálcio e fosfato, a partir do espaço extracelular da medula óssea para a camada osteóide (Caverzasio & Bonjour, 1996). A membrana plasmática dos osteoblastos é rica em fosfatase alcalina, a qual entra na circulação sanguínea. A concentração plasmática desta enzima é usada como um marcador bioquímico da formação óssea. Ao final da produção da matriz óssea e deposição dos íons minerais, os osteoblastos tornam-se células de revestimento achatadas ou osteócitos (Nijweide et al., 1996). Um lento processo de deposição mineral (mineralização secundária) completa o processo de formação óssea (Meunier & Boivin, 1997).

Osteócitos são de longe o tipo de célula mais comum no osso (Mullender et., 1996) originam a partir de osteoblastos embebidos na matriz orgânica óssea, a qual se torna subsequentemente mineralizada. Os osteócitos possuem processos celulares longos e numerosos formando uma rede de finos canalículos que os conecta com osteoblastos ativos e as células de revestimentos achatadas. O fluido proveniente do espaço extracelular na medula óssea circula nesta rede. Osteócitos provavelmente exercem uma função na homeostasia deste fluido extracelular e na ativação local da formação e/ou reabsorção óssea em resposta a cargas mecânicas (Nijweide, 1996).

Osteoclastos são células gigantes com o predomínio de células multinucleadas contendo de 4-20 núcleos e sua função é absorver o osso. Osteoclastos são importantes para o balanço do cálcio e o remodelamento do esqueleto, mas também podem ser importantes para a manutenção da qualidade óssea (Burr, 2002). Posteriormente a reabsorção, a formação óssea é iniciada, o que indicaria que alguns aspectos da atividade osteoclástica são importantes para a formação óssea.

Uma convincente evidência deste fato é que, no esqueleto adulto normal, a formação óssea é quase exclusivamente iniciada em áreas que sofreram a reabsorção óssea (Hattner et al., 1965; Martin & Sims, 2005). Consistente com isto, o número de núcleos de um osteoclastos tem sido correlacionado com o número de osteoblastos (Thompson et al., 1975), indicando eventos de sinalização local entre osteoclastos e osteoblastos. Tradicionalmente, um arranjo de fatores de crescimento e citocinas derivadas da reabsorção óssea, incluindo a superfamília TGF-β e IGFs (Mundy & Bonewald, 1990; Baylink et al., 1993) são conhecidas por mediar a ativação de osteoblastos levando a formação óssea. Os osteoclastos originam a partir de células troncos hematopoiéticas, provavelmente da linhagem mononuclear/fagocítica (Suda et al., 1996), e são encontrados em contato com a superfície óssea calcificada dentro de cavidades denominadas lacunas de Howship (também conhecidas como lacunas de reabsorção) que resulta a partir de sua atividade absortiva. A reabsorção osteoclástica ocorre na interface células/osso em um microambiente isolado (Teitelbaum et al., 1996; Baron, 1996). Ao considerar estes fatos, a mais proeminente característica estrutural dos osteoclastos é a profunda borda da membrana plasmática, chamada de borda em escova, na área oposta a matriz óssea. Esta estrutura é circundada através de um anel periférico firmemente aderido a matriz óssea, o qual isola o compartimento de reabsorção sub-osteoclástica. Os mecanismos de reabsorção envolvem a secreção de íons hidrogênio e enzimas proteolíticas (Teitelbaum et al., 1996; Baron, 1996) dentro do compartimento de reabsorção sub-osteoclástico. Estas enzimas as quais incluem colagenases e catepsinas são responsáveis pela degradação da matriz orgânica. O processo libera os minerais que contribuem para a homeostase do cálcio e do fósforo. Desta forma, marcadores bioquímicos da degradação do colágeno, tais como moléculas interligadoras como a hidroxiprolina e piridinolina, são encontradas no plasma e na urina e podem prover estimativas da taxa de reabsorção óssea (Eyre, 1996, Garnero & Delmas, 1998).

1.3 Fisiologia

Tanto a forma quanto a estrutura do osso são continuamente renovadas e modificadas através dos processos de modelamento e remodelamento.

1.3.1 Modelamento ósseo

O modelamento ósseo começa com o desenvolvimento do esqueleto durante a vida fetal e continua até o final da segunda década de vida, quando o crescimento longitudinal do esqueleto está completado. No processo de modelamento, o osso é formado em posições que diferem a partir dos locais de reabsorção, levando a mudanças na forma ou microarquitetura do esqueleto. O crescimento longitudinal de um osso longo típico, tal como a tíbia, depende da proliferação e diferenciação das células da cartilagem na placa de crescimento epifisial. Crescimento da seção transversal, tal como o aumento na circunferência da diáfise radial, ocorre como novo osso formado abaixo do periósteo. Simultaneamente o osso é reabsorvido na superfície endosteal. O modelamento ósseo poderia continuar, mas a um grau menos intenso, durante a vida adulta quando a reabsorção na extremidade da superfície endosteal aumenta a tensão mecânica na cortical óssea remanescente, levando a estimulação da aposição óssea periostal. Este fenômeno, o qual aumenta com o envelhecimento e é mais pronunciado em homens do que nas mulheres, compensa em parte os efeitos negativos da força mecânica que leva a reabsorção óssea da superfície endosteal (Kanis, 1994; Einhorn, 1996; Fleisch, 1997).

1.3.2 Remodelamento ósseo

O remodelamento ósseo ocorre simultaneamente com o modelamento a partir da vida fetal e durante a maturidade esquelética, quando se torna o processo predominante que ocorre por toda a vida adulta. O remodelamento ósseo é um processo essencial envolvido tanto na manutenção da qualidade quanto da resistência óssea, como também na homeostasia do cálcio (Seeman & Delmas, 2006). Dois tipos de remodelamento teem sido sugeridos, cada um com um diferente

propósito (Burr, 2002; Parfitt, 2002). O primeiro tipo é estocástico, o qual é primariamente dedicado à manutenção da homeostasia do cálcio, e esta sob o controle hormonal (Noble, 2003). O segundo tipo é focado na remoção de microdanos, e então essencial para as propriedades mecânicas do esqueleto (Burr, 2002; Parfitt, 2002).

Em adultos saudáveis o remodelamento é um continuo processo fisiológico, uma vez que a formação óssea é precedida pela reabsorção óssea, o que promove um completo preenchimento da matriz óssea removida com um tecido ósseo recém sintetizado (Hattner et al., 1965; Takahashi et al., 1964).

A reabsorção e a formação óssea ocorrem no mesmo local, de modo que não ocorra nenhuma mudança na forma do osso. Este processo constante de turnover permite ao esqueleto liberar fosfato de cálcio se a absorção intestinal deste mineral é menor do que a quantidade excretada na urina (Broadus, 1996).

No esqueleto adulto, aproximadamente 5-10% do osso existente é substituído a cada ano através do remodelamento. Isto não ocorre uniformemente pelo esqueleto, mas em pontos focais ou discretos. A estrutura morfológica dinâmica do turnover é a “unidade básica multicelular” (BMU), também chamada de “unidade de remodelamento ósseo” (BRU). Esta entidade morfológica formada quando o processo está terminado é chamada de “unidade estrutural óssea” (BSU) (Parfitt, 1992). A BSU corresponde a um “pacote” no osso esponjoso, e a um ósteon na cortical óssea. Em ambos os ossos corticais e esponjosos, o processo de remodelamento começa com a reabsorção óssea através dos osteoclastos. Esta fase é finalizada dentro de poucos dias e é seguida pela retirada dos osteoclastos multinucleados e uma fase de reversão (Figura 1).

Na fase de reversão, células mononucleares alinham-se junto à lacuna de reabsorção e depositam uma linha de cemento marcando o limite da erosão prévia e o osso recentemente formado. Estas células mononucleares são subsequentemente substituídas por

células osteoprogenitoras, as quais se diferenciam em osteoblastos de formato cuboidal. A matriz orgânica é então depositada, seguida pela deposição de minerais. As lacunas são gradualmente preenchidas com novo osso durante vários meses. Este processo de reabsorção óssea seguida pela formação de osso no mesmo local é denominada "coupling" (Mundy et al., 1996; Rodan, 1996; Martin & Udagawa, 1998).

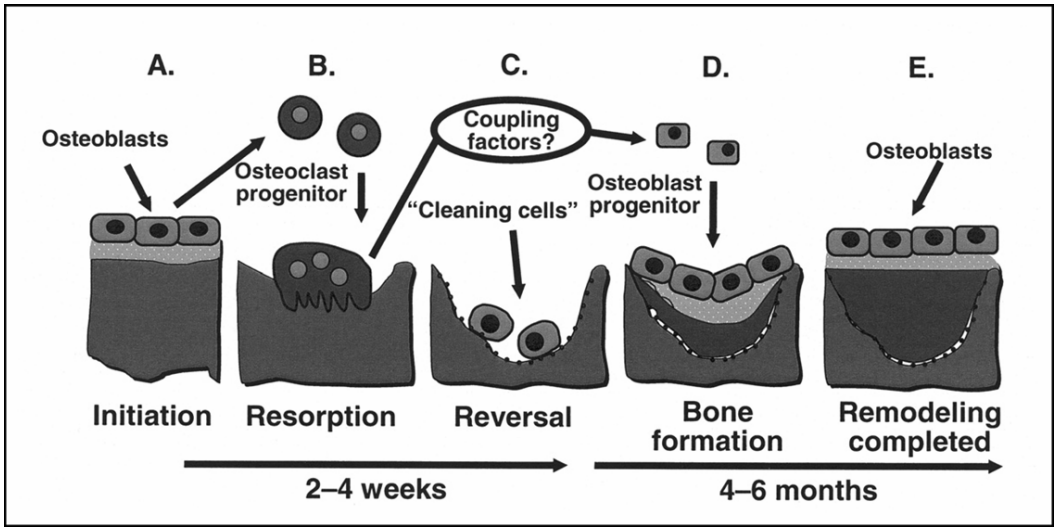

Figura 1. O ciclo do remodelamento ósseo. Remodelamento do osso começa em unidades multicelulares com a ativação osteoblástica, a qual promove a diferenciação, fusão e ativação de osteoclastos (A e B). Quando as lacunas de reabsorção são formadas, os osteoclastos deixam as áreas e células mononucleares de origem indefinida aparecem para promover a "limpeza" dos remanescentes da matriz orgânica deixadas pelos osteoclastos, além de possivelmente formar a linha de cemento (linha pontilhada) na parte inferior da lacuna (C). Durante o processo de reabsorção, fatores de acoplamento, incluindo fatores de crescimento semelhantes à insulina e fatores de crescimento transformante, são liberados a partir da matriz óssea extracelular, e estes fatores de crescimento contribuem para o recrutamento de osteoblastos para as lacunas de reabsorção e sua ativação (D). Os osteoblastos preencherão a lacuna óssea com novo osso; quando a mesma quantidade de osso formada é semelhante ao volume reabsorvido o processo de remodelamento é finalizado, e a matriz extracelular mineralizada é então coberta através do osteóide e uma monocamada celular de osteoblastos (E). (Lerner et al., 2006)

O processo de remodelamento é controlado através de citocinas produzidas sistêmica ou localmente (Mundy et al., 1996; Rodan, 1996; Martin & Udagawa, 1998; Manolagas & Jilka, 1995). A manutenção de uma massa esquelética normal, saudável e competente mecanicamente

dependerá da preservação do equilíbrio entre o processo de reabsorção e formação óssea. Falhas na coordenação entre reabsorção e a formação óssea resulta numa perda líquida de osso. Isto é o que ocorre na osteoporose, caso resulte de uma deficiência de hormônios sexuais, hiperparatireoidismo primário, hipertireoidismo ou exposição endógena ou exógena a excesso de glicocorticóides.

1.3.3 Comunicação entre osteoblastos e osteoclastos

A formação de osteoclastos é controlada através de hormônios circulantes, incluindo o hormônio da paratireóide 1α,25-dihidroxicholecalciferol (calcitriol), e os hormônios esteróides gonadais, estrogênio e testosterona (Martin & Udagawa, 1998). O microambiente da medula óssea exerce uma função essencial como fonte de citocina tal como o fatores de necrose tumoral (TNFs) e interleucinas (Horowitz, 1993; Jilka, 1998), as quais também regulam a formação e atividade de osteoclastos. Estes fatores locais e sistêmicos regulam a formação e atividade de osteoclastos.

Hormônios e citocinas atuam na linhagem osteoblástica, as quais possuem na sua superfície celular moléculas conhecidas como ligante RANK (RANKL, formalmente conhecido como fator de diferenciação osteoclástica, TRANCE), e um receptor na superfície celular, osteoprotegerina (Suda et al., 1999). RANKL é um membro dos ligantes da família TNF, o qual esta presente nas células da linhagem osteoblástica e interage com os precursores osteoclásticos provenientes da linhagem hematopoiética. Esta interação promove a diferenciação e fusão de precursores osteoclásticos, então levando a formação de osteoclastos maduros. Osteoprotegerina é um membro solúvel da superfamília de receptores TNF que é produzido pela linhagem osteoblástica e inibe a formação de osteoclastos (Suda et al., 1999).

1.3.4 Mecanismos de ação hormonal

Calcitonina inibe a reabsorção óssea atuando diretamente nos osteoclastos maduros (Martin et al., 1996). Bisfosfonatos, os quais são usados no tratamento da osteoporose, também inibem os osteoclastos, provavelmente por interferirem com o sistema de comunicação entre osteoblastos e osteoclastos (Fleisch, 1997). Eles também reduzem o número de osteoclastos por inibirem o seu recrutamenteo ou sua sobrevida. Estrogênio e provavelmente testosterona exercem seus efeitos na reabsorção óssea através de inibição da produção de citocinas, particularmente TNFs, interleucina-1 e interleucina-6 (Horowitz, 1993; Jilka, 1998; Ammann et al., 1997; Ducy & Karsenty, 1998).

1.3.5 Fatores de crescimento

A formação de osteoblasto requer fatores de transcrição chamados de cbfa1 e osf2, os quais controlam a diferenciação de osteoblastos e a formação de osso durante o desenvolvimento esquelético e, também, a função de amadurecimento de osteoblastos diferenciados (Ducy et al., 1997; Ducy et al., 1999). Vários fatores de crescimento, incluindo os fatores de crescimento semelhantes à insulina (IGFs), fator de crescimento transformante-β, fator de crescimento fibroblástico, fator de crescimento derivados de plaquetas, proteínas morfogenéticas e prostaglandinas podem estimular a proliferação de osteoblastos *in vitro* (Canalis, 1996). Contudo, a relevância de tais fatores de crescimento para a proliferação e diferenciação osteoblástica *in vivo* ainda não esta clara. Não obstante, tem sido sugerido que a produção e ação de fatores de crescimento são vitais para a estimulação da formação óssea em resposta aos hormônios sistêmicos, tais como o hormônio da paratireóide (PTH), agentes osteogênicos, tais como fluoreto e tensão mecânica (Rodan, 1996).

2 Osteoblastos

2.1 Origem dos osteoblastos

Um dos correntes dogmas da biologia óssea é que os osteoblastos maduros são células diferenciadas provenientes de células precursoras presente na medula óssea. A partir do trabalho pioneiro de Friedenstein e colaboradores (Friedenstein et al., 1978; Friedenstein, 1991) têm sido reconhecido que compartimentos não-hematopoiéticos da medula óssea (também conhecido como estroma da medula óssea) contêm um grupo de células semelhantes a fibroblastos com potencial de diferenciação em linhagem osteogênica (referidas como células-tronco mesenquimais ou células do estroma da medula óssea, células-tronco esqueléticas). A exata localização destas células-tronco ainda não é conhecida, mas recentes trabalhos sugerem que as células-tronco mesenquimais estejam localizadas nos espaços perivasculares como células subendoteliais ao redor dos sinusóides capilares na medula óssea (Sacchetti et al., 2007). Entretanto, ainda não é conhecido como as células-troncos são recrutadas e ganham acesso as regiões formadoras de tecido ósseo.

2.2 Mudanças no potencial de diferenciação osteogênica de células-tronco mesenquimais durante o envelhecimento

Um dos mais consistentes achados histomorfométricos em biópsias ósseas obtidas a partir de pessoas idosas é a presença da diminuição da espessura das paredes ósseas trabecular e cortical indicando redução da capacidade de formação óssea pelos osteoblastos durante o remodelamento (Brockstedt et al., 1993). A espessura das paredes ósseas é dependente tanto do número de osteoblastos recrutados no começo da fase de formação óssea, como também da atividade dos osteoblastos individuais. O recrutamento de número adequado de osteoblastos é dependente da disponibilidade de células-tronco e precursoras, assim como de suas propriedades em responder a sinais de proliferação, diferenciação e sinais quimiotáticos no microambiente ósseo. As funções de produção de matriz e mineralização dos osteoblastos maduros são dependentes das propriedades em responder a hormônios, fatores de crescimento e citocinas

como também da disponibilidade de nutrientes e íons necessários para realização destes processos. O comprometimento da função osteoblástica relacionado à idade pode ser resultado de algum mecanismo que interfira nestes processos. Stenderup et al. (2001) têm relatado que o número de células-tronco mesenquimais não muda com o envelhecimento ou na osteoporose. Entretanto, células-tronco mesenquimais exibem limitado tempo de meia-vida *in vitro* e todas as características de um fenótipo senil (Stenderup et al., 2003). Outros fatores comprometem a proliferação celular de células-tronco mesenquimais com o envelhecimento, como por exemplo, o próprio microambiente senil, o qual pode afetar as funções das células-tronco mesenquimais (Abdallah et al., 2006). Portanto, o envelhecimento intrínseco das células-tronco mesenquimais e os efeitos negativos do microambiente senil são possíveis mecanismos a serem considerados nos defeitos das funções osteoblástica associada à idade na formação óssea *in vivo* em humanos.

O estudo dos mecanismos da diferenciação de osteoblastos é uma das áreas centrais de pesquisa na osteoporose. É esperado que o entendimento dos mecanismos moleculares e celulares que controlam a diferenciação de osteoblastos proverá novas abordagens para o tratamento de doenças ósseas degenerativas incluindo osteoporose. Em adição, o estudo da biologia osteoblástica proviria novas perspectivas dos processos biológicos básicos que controlam e mantém o turnover ósseo.

2.3 Cultura de células osteoblásticas

Desde que técnicas têm se tornado disponíveis para a isolação e análise de células precursoras osteogênicas houve um considerável interesse no uso destes sistemas de culturas para entender os eventos determinantes que regulam a parada da divisão celular e o início dos eventos que são os marcadores da diferenciação. Tanto em culturas primárias de osteoblastos (Gerstenfeld et al., 1987; Aronow et al., 1990; Gronowicz et al., 1989; Lian & Stein, 1992), como também na linhagem clonada de células de calvária de camundongos MC3T3-E1 (Sudo et al. 1983;

Franceschi & Iyer, 1992; Quarles et al., 1992), um período de rápida divisão celular é seguido por um período de transição que é caracterizado pela deposição de matriz extracelular insolúvel rica em colágeno tipo I e um aumento inicial na atividade de fosfatase alcalina. Tardiamente, um terceiro estágio ocorre, começando aproximadamente 2 semanas após o cultivo celular, o qual é caracterizado por um aumento significativo na atividade da fosfatase alcalina, expressão de fosfatase alcalina e deposição mineral (Figura 2)

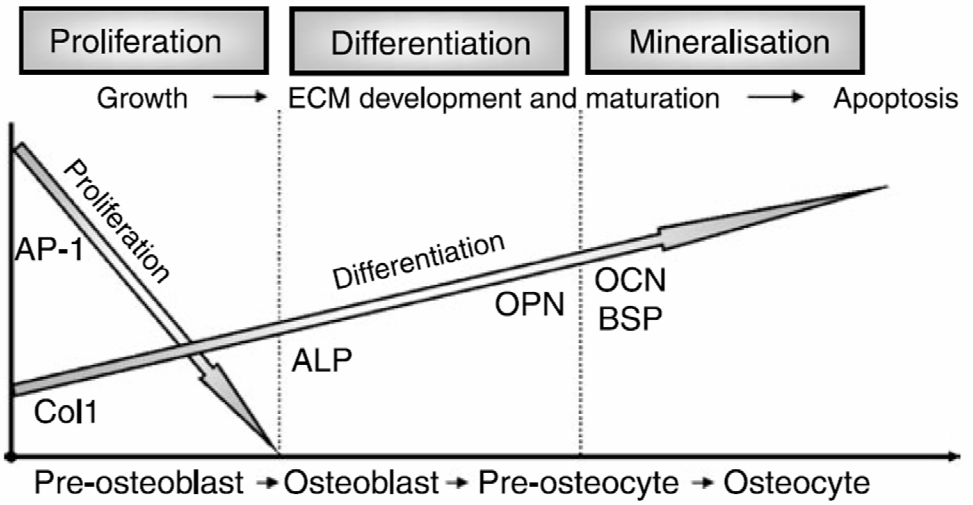

Figura 2. Principais fatores produzidos pelos osteoblastos e usados como marcadores dos diferentes estágios de desenvovimento da diferenciação osteoblástica (adaptado a partir de Stein & Lian, 1993). ECM, matriz extracelular; AP-1, proteína ativador-1. Col1, colágeno tipo 1, ALP, fosfatase alcalina; OPN, osteopontina, OCN, osteocalcina; BSP, sialoproteína óssea (Lian & Stein, 1992).

Há evidência *in vitro* e *in vivo* que a deposição de uma matriz de colágeno no estágio de transição poderia regular ou ao menos ter um efeito permissível no terceiro estágio da maturação do fenótipo osteoblástico. De fato, o cultivo as células precursoras osteoblásticas na ausência de ascorbato, o qual é necessário para a deposição de colágeno, resulta no crescimento celular, mas não induz um processo de diferenciação efetivo (Gerstenfeld et al., 1987; Franceschi and Iyer, 1992; Quarles et al., 1992; Franceschi et al., 1994). Em adição, a hipótese de que a interação

célula-colágeno tipo I exerce função na maturação osteoblástica é suportada através da análise do tecido ósseo de indivíduos com desordens hereditárias como a osteogênese imperfeita, onde as células secretam colágeno tipo I com defeitos na estrutura e/ou diminuição na quantidade.

2.4 MC3T3-E1, um modelo "in vitro" para o desenvolvimento ósseo

MC3T3-E1, uma linhagem celular imortalizada espontaneamente foi estabelecida a partir de células da calvária de camundongos recém-nascidos e selecionadas baseadas na alta atividade de fosfatase alcalina no estado de repouso (Kodama et al., 1981). As células MC3T3-E1 têm demonstrado ter capacidade de diferenciar-se em osteoblastos e formar uma matriz óssea mineralizada e nodular, um processo que é semelhante à ossificação intramembranosa (Quarles et al., 1992; Sudo et al., 1982). Este sistema de cultura de osteoblastos *in vitro* é biologicamente relevante, pois desenvolve uma seqüência temporal de fenômenos associados com a expressão de genes que codificam os marcadores de fenótipo osteoblástico seguindo padrões específicos de expressão gênica e distribuição celular de forma semelhante ao observado durante o desenvolvimento da calvária dos fetos *in vivo*.

O processo através do qual a matriz extracelular nodular é formada e mineralizada é complexo e nas células MC3T3-E1 pode ser observado em três distintas fases, denominadas proliferação, diferenciação e mineralização (Choi et al., 1996; Quarles et al., 1992; Sudo et al., 1983). Cada um destes passos envolve a expressão minuciosamente regulada de genes relacionados ao osso e fatores de transcrição. Durante os primeiros 10 dias, as células MC3T3-E1 sofrem uma ativa proliferação (Quarles et al., 1992). Este período de desenvolvimento osteoblástico é caracterizado pela expressão de muitos genes relacionados com o ciclo celular e a proliferação (c-fos, c-myc e histonas) como também genes da matriz extracelular tais como colágeno tipo I (Choi et al., 1996). O período entre 10-20 dias marca a segunda fase do desenvolvimento dos osteoblastos. Neste estágio a taxa de proliferação dos pré-osteoblastos

diminui ao mesmo tempo em que estas células começam a se diferenciar em osteoblastos (Quarles et al., 1992). Durante este estágio ocorre a formação e maturação da matriz extracelular, a qual é marcada através da elevação na expressão de proteínas de matriz extracelular tais como colágeno tipo I, fibronectina, TGFβ1 e osteonectina (Choi et al., 1996). Outras proteínas expressas durante a fase de diferenciação incluem a fosfatase alcalina e a osteopontina (Quarles et al., 1992; Vary et al., 2000). A terceira e última fase do desenvolvimento do osteoblasto é a mineralização da matriz extracelular. Este estágio é marcado pela expressão de osteocalcina, sialoproteína óssea e osteopontina, proteínas envolvidas no processo de mineralização (Choi et al., 1996). Estas mudanças temporais na expressão de genes relacionados ao osso durante os estágios de desenvolvimento da MC3T3-E1 são paralelos aos observados *in vivo* (Quarles et al., 1992; Sudo et al., 1983). Portanto, as células MC3T3-E1 provêm um excelente modelo para estudar os mecanismos de ação de compostos com uso potencial no tratamento de doenças ósseas degenerativas e suas influências nos processos de formação e diferenciação óssea (Figura 3).

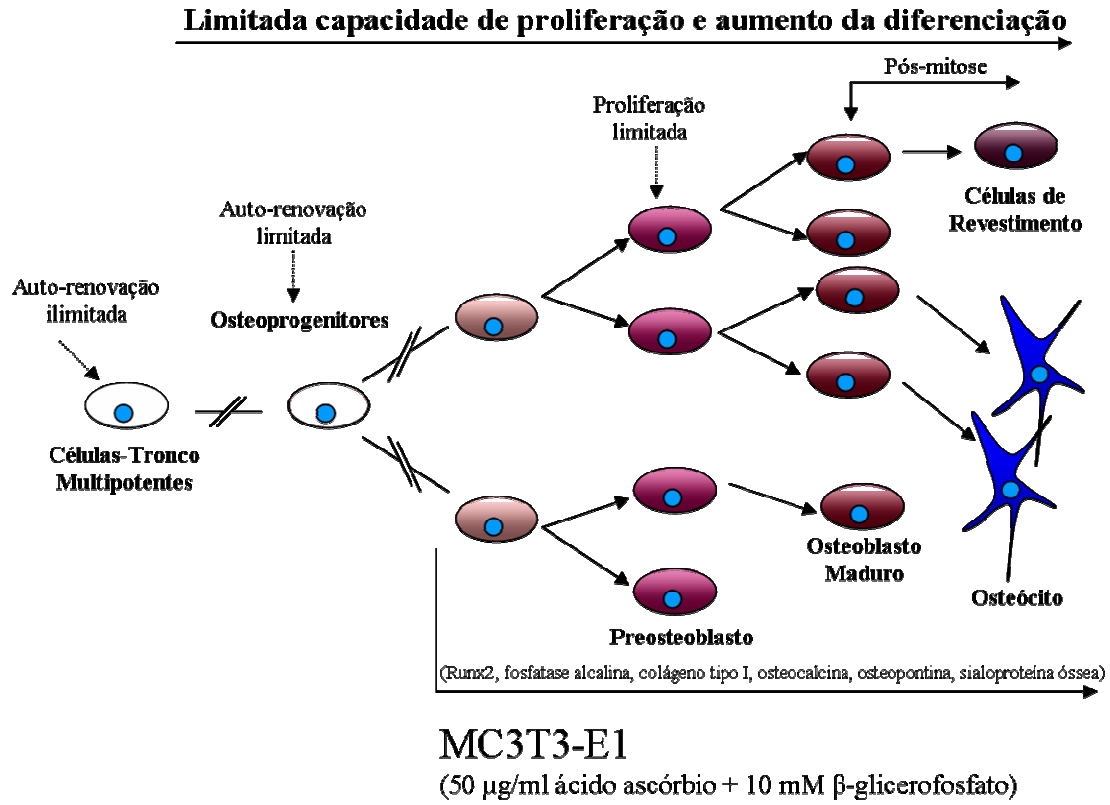

Figura 3. Sequência presumida de eventos envolvidos na diferenciação de osteoblastos começando a partir da população de células pluripotentes. As células osteoprogenitoras referem-se a população de osteoprogenitores maduros e imaturos.

3 Osteoporose

3.1 Definição

A osteoporose, doença caracterizada por diminuição global da massa óssea e deterioração estrutural do tecido ósseo, leva a fragilização dos ossos e aumento da susceptibilidade a fraturas, especialmente de quadril, espinha dorsal e punho. A osteoporose resulta de envelhecimento natural, impedimento de desenvolvimento de pico de massa óssea (por puberdade retardada ou desnutrição) ou excessiva perda óssea na fase adulta, fruto de deficiência estrogênica na mulher, desnutrição ou uso de corticosteróides (NIH, 2001; WHO, 2003). A osteoporose vem sendo mundialmente considerada um dos principais problemas de saúde pública, devido a repercussões individuais (mortalidade, morbidade, incapacidade funcional) e sociais (diminuição da força de

trabalho, aumento do risco de institucionalização, ônus econômico) (Fuller, 2000; Cummings & Melton, 2002). As fraturas de quadril são consideradas as mais sérias, induzindo hospitalização, letalidade em torno de 20% e incapacidade permanente em 50% dos casos. Em 1990, havia 1,7 milhões de fraturas de quadril isoladas no mundo. Com o crescimento demográfico, estima-se seu aumento para seis milhões até 2050 (WHO, 2003).

É bem determinado que o volume de massa óssea é mantido por duas fases do remodelamento ósseo, denominadas de formação óssea pelos osteoblastos e reabsorção óssea pelos osteoclastos (Rodan et al., 1992). A redução da massa óssea é, portanto, devido a um aumento da reabsorção e/ou uma redução da formação óssea. Fatores nutricionais e farmacológicos poderiam prevenir a perda óssea com o aumento da idade (Bonjour et al., 1996). Um desafio na pesquisa nutricional é identificar componentes da alimentação que contribuam para a manutenção da saúde óssea. A qualidade óssea descreve aspectos da composição e estrutura óssea que contribuem para a força óssea independentemente da densidade mineral. Estes aspectos incluem o turnover ósseo, microarquitetura, mineralização, microdanos e a composição orgânica e mineral da matriz óssea. Novas técnicas para avaliar estes componentes da qualidade óssea têm sido desenvolvidas e produzirão importantes perspectivas na determinação dos riscos de fraturas em casos de doenças tratadas e não tratadas (Compston, 2006; Liacata, 2009).

Substanciais avanços no tratamento da osteoporose têm sido obtidos nas últimas duas décadas. Embora uma grande variedade de drogas seja disponível atualmente, o tratamento da osteoporose é primariamente baseado em agentes anti-reabsortivos incluindo estrogênios, raloxifeno (um modulador seletivo de receptores de estrogênio), o grupo dos bisfosfonatos (alendronato, residronato, ibandronato e zoledronato), calcitonina e ranelato de estrôncio. Embora os mecanismos de ação difiram dentre e entre estas classes de drogas, a inibição da reabsorção

óssea mediada por osteoclastos pode ser considerada uma via final comum (Ettinger, 2003; Rosen, 2005).

Em contraste aos agentes anti-reabsortivos acima, os agente anabólicos são um novo e importante avanço no tratamento da osteoporose porque eles aumentam a formação óssea. O hormônio recombinante intacto da paratireóide (hrPTH 1- 84; Preotact) e um peptídeo humano recombinante do PTH 1-34 (Teriparatide) são pertencentes a este grupo de agentes anabólicos (Pleiner-Duxneuner et al., 2009).

3.1 Osteoporose e Estresse Oxidativo

Estudos têm revelado a ligação entre antioxidantes e a saúde óssea (Maggio et al., 2003; Zhang et al., 2006). Superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX) são as principais enzimas antioxidantes envolvidas na proteção contra O^{2-} e H_2O_2. A despeito das evidências ligando a perda óssea e o estresse oxidativo, estudos que avaliam a atividade de enzimas antioxidantes em casos de doenças ósseas em humanos são limitados (Maggio et al., 2003; Sontakke et al., 2002; Yalin et al., 2005). Em humanos o H_2O_2 é metabolizado através da CAT e GPX. Embora, ambas removam o mesmo substrato, somente a GPX pode remover efetivamente os hidroperóxidos orgânicos, tornando-se a principal fonte de proteção contra baixos níveis de estresse oxidativo (Matés et al., 1999).

Muitos estudos sugerem que ROS tem a principal função na osteoporose pós-menopausa. Dreher et al., (1998) declarou que a falta ou diminuição da expressão da GPX poderia resultar no prejuízo da função de osteoblastos e, consecutivamente, no desenvolvimento de doenças ósseas tais como a osteoporose. Sontakke & Tare (2002) e Maggio et al. (2003) têm relatado que a atividade da GPX no plasma é significativamente menor nas mulheres com osteoporose pós-menopausa. Enzimas antioxidantes são consideradas como marcadores dos mecanismos de defesa antioxidante para a reabsorção óssea (Garrett et al., 1990).

Trabalhos demonstram que o estado de saúde geral do osso melhorou com prolongados períodos de suplementação antioxidante, os quais podem ser utilizados como tratamentos paliativos para osteoporose. O aumento da incorporação dietética da riboflavina e piridoxina tem sido associado com maiores densidades minerais ósseas em mulheres e homens idosos (Yazdanpanah et al., 2007). Suplementação vitamínica antioxidante durante a senilidade tem direcionado atenção ao fato de que a piridoxina e certamente outras vitaminas do complexo B (riboflavina, folato e cobalamina) funcionam como cofatores de certas enzimas que mantêm os níveis de homocisteínas baixos (Nygard et al., 1998; Yazdanpanah et al., 2008; Yazdanpanah et al., 2007), o qual é considerado um novo e potencial fator de risco modificador para as fraturas osteoporóticas relacionadas a idade, principalmente em mulheres homozigotas para o alelo C677T do gene da enzima metilenotetraidrofolato redutase (Yazdanpanah et al., 2008).

4 Riboflavina

Riboflavina (Fig. 4), também conhecida como Vitamina B2 (nome sistemático 7,8-dimetil-10-(D-1'-ribitil)-isoaloxazina) é um micronutriente facilmente absorvido com função chave na manutenção da saúde em humanos e animais. Leite, queijo, vegetais verdes folhosos, fígado, rins, feijões, fermentos, cogumelos e amêndoas são boas fontes de riboflavina. O nome "riboflavina" tem origem a partir de "ribose" e "flavina". Como a maioria das vitaminas hidrossolúveis, estudos têm demonstrado que sistemas de transportes específicos estão envolvidos para facilitar a eficiente entrada de riboflavina através da membrana celular (Said et al., 1998; Huang and Swaan, 2001). Análises das concentrações basais no plasma demonstraram uma concentração de 12,6 nmol riboflavina/L (8,2-20,0 nmol/L) e 78,9 nmol flavocoenzimas/L (62,2-96,5 nmol/L) (Zempleni et al., 1996). As concentrações basais no plasma de FAD foram estimadas constituir 75,5% das flavoenzimas (Ohkawa et al., 1982). Deficiência fisiológica da riboflavina promove manifestações clínicas de doenças associadas com o retardo do crescimento,

anemia, doenças cardiovasculares, desordens neurodegenerativas, dermatite seborréica, glossite e queilose (Cooperman & Lopez, 1991; Powers, 2003).

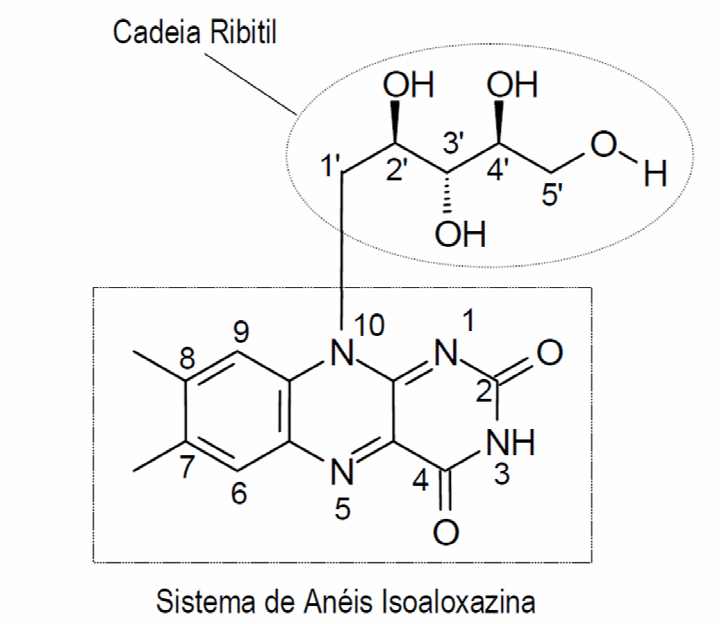

Figura 4. Estrutura da riboflavina. Em destaque, a cadeia ribitil e o sistema de anéis isoaloxazina que apresenta o sistema π conjugado.

Desde a descoberta e caracterização química a riboflavina tem sido correlacionada com diferentes processos celulares envolvendo transferência de elétrons e participação na oxidação de vários substratos orgânicos na cadeia de transporte de elétrons presente na mitocôndria, bem como nas reações envolvendo o complexo citocromo P450 (Massey, 2000). Na literatura constam várias citações sobre o papel biológico desta vitamina:

4.1 Quimioprotetor

Webster et al. (1996) demonstraram que a deficiência da riboflavina na dieta provocou um aumento da expressão de enzimas de reparo do DNA (poli-ADP-ribose polimerase, DNA polimerase β e DNA ligase), quando ratos deficientes de riboflavina eram tratados com carcinógenos, enquanto que a suplementação com riboflavina provocou uma diminuição da expressão destas enzimas, sugerindo um menor grau de danos no DNA.

4.2 Cofator de enzimas

Riboflavina é o precursor de flavocoenzimas essenciais, flavina mononucleotídeo (FMN) e flavina dinucleotídeo (FAD). FAD e FMN são grupos prostéticos de numerosas enzimas que catalisam as várias reações de transferência de elétrons que ocorrem durante a produção de energia, biosíntese, desintoxicação e vias de capturas de elétrons (McCormick et al., 1989). Portanto, a riboflavina é importante para a atividade de oxidases e desidrogenases presentes em vias metabólicas relacionadas com produção de energia, metabolismo de proteínas e ácidos graxos. Além do mais, a riboflavina está envolvida nas transformações metabólicas de algumas vitaminas como o ácido fólico (Pearson and Turner, 1975), piridoxina (Rasmussen et al., 1980), vitamina K (Preusch and Suttie, 1981) e niacina (Pekkanen and Rusi, 1979).

4.3 Citotóxica

Edwards et al. (1994 e 1999) demonstraram que os fotoprodutos obtidos a partir do ácido indol-acético na presença da riboflavina induziram a morte por apoptose das células HL60 e das células NOS/2 (tumor murino). Nosso grupo tem demonstrado com detalhes os mecanismo de indução de morte de células leucêmicas (Souza et al. 2006) e células de câncer de próstata (de Souza Queiroz et al., 2007) após tratamento com riboflavina irradiada.

4.4 Indutor de diferenciação

Dados inéditos obtidos recentemente por nosso grupo de pesquisa revelaram que a riboflavina em concentrações na faixa de nM causou diferenciação de células da leucemia mielóide humana (Ferreira et al., 2005 TI0505220-3 - Patente Brasileira).

4.5 Oxidante e antioxidante

As flavinas podem contribuir para o estresse oxidativo por produzirem superóxido, mas também podem reduzir hidroperóxidos e outros radicais de oxigênio (Massey, 2000). Minami et al. (1999) demonstraram que a citotoxicidade da riboflavina irradiada sobre fibroblastos seria

devido à produção de peróxido de hidrogênio. Além de estar envolvida com a produção de energia corporal, a riboflavina também exerce função como antioxidante através do seqüestro de partículas deletérias no corpo conhecidas como radicais livres. Esta sua função é associada com uma enzima denominada glutationa redutase, a qual ajuda manter os níveis de glutationa (GSH), o principal protetor contra os efeitos deletérios dos radicais livres, atuando como um sequestrador e cofator na desintoxicação metabólica de espécies reativas de oxigênio (Bray et al., 1993). A glutationa redutase é constantemente regenerada através das coenzimas NAD e FAD, as quais contêm niacina e riboflavina, respectivamente (Murray, 1996; Rivlin, 1996). Glutatione peroxidase (GPX) usa GSH como cofator para reduzir moléculas de H_2O_2 em H_2O e hidroperóxidos orgânicos em álcoois (Matés, 2000; Ray and Husain, 2002), enquanto a glutationa S-transferase (GST) catalisa a conjugação de GSH com produtos da peroxidação lipídica e substratos xenobióticos na desintoxicação celular.

4.6 Fotoprodutos da Riboflavina

Importantes avanços com relação ao efeito da radiação na química da riboflavina têm sido alcançados, demonstrando que as flavinas são degradadas através de uma variedade de reações destacando-se a fotólise (fotoredução intramolecular) e fotoadição (fotoadição intramolecular) como as principais reações (Ahmad et al., 2004; Holzer et al. 2005). A fotodegradação da riboflavina em soluções aquosas resulta em um número de fotoprodutos tais como 7,8-dimetil-10-(formilmetil) isoaloxazina, lumicromo and lumiflavina (Figura 5).

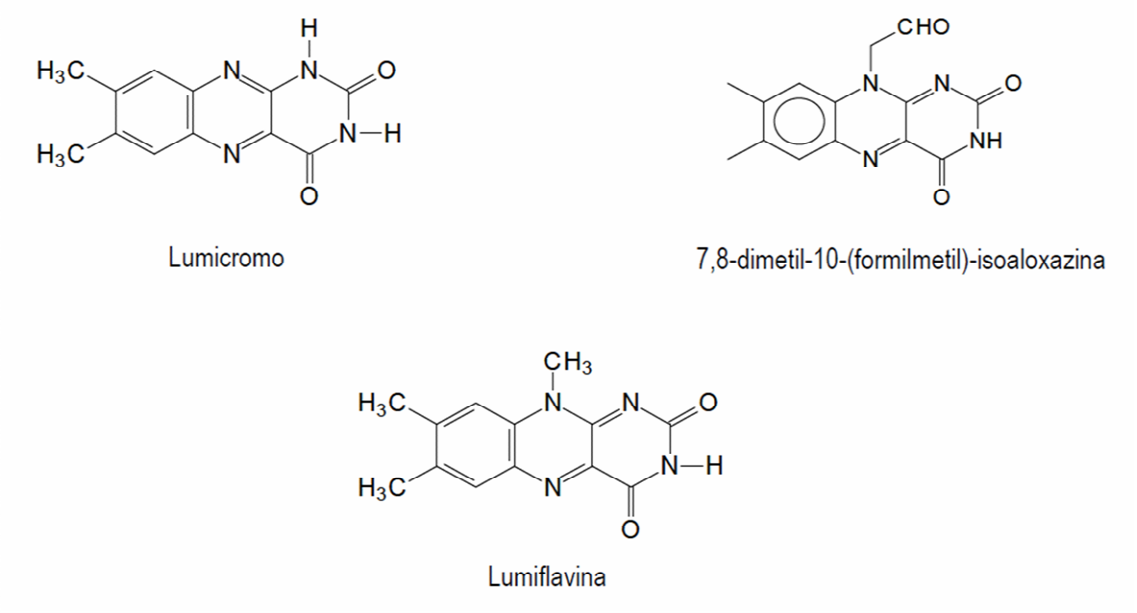

Figura 5. Estrutura química dos principais fotoprodutos da riboflavina.

Em nosso grupo a porcentagem de riboflavina e seus fotoprodutos têm sido determinados através da espectrometria de massa após radiação de uma solução estoque de riboflavina a 250 µM diluído em meio de cultura. A distribuição dos produtos formados foi apresentada da seguinte maneira: 79% riboflavina, 6,2% de lumicromo, e 14,8% de formilmetilflavina, lumiflavina, 2-cetoriboflavina e 4-cetoriboflavina (de Souza Queiroz et al., 2007). É conhecido que as diferentes flavinas exibem importantes diferenças no seu comportamento farmacocinético. Riboflavina, lumiflavina e ésteres de cadeia curta são rapidamente removidos a partir do soro, e recuperados em quantidades comparáveis a partir do fígado e rins (Edward set al., 1999). O aumento na hidrofobicidade das moléculas fotosensibilizadas é conhecido por aumentar a sua afinidade por tumores e outros tipos de células hiperproliferantes (Kessel, 1989). Recentes estudos de nosso grupo têm definido em detalhes os mecanismos pelos quais os fotoprodutos da RF induzem apoptose de células leucêmicas mielóides e células de câncer de próstata (Souza et al., 2006; Queiroz et al., 2007). No entanto os mecanismos moleculares que envolvem a incorporação dos fotoprodutos da riboflavina ainda são pouco conhecidos. Derivados da

riboflavina incluindo FMN, FAD, lumiflavina e lumicromo demonstraram significante inibição da incorporação da riboflavina como observado em ensaios de competitividade (Huang et al., 2001; Said et al., 2000; Zempleni and Mock, 2000; Kumar et al., 1998; Said & Ma, 1994). Como abordado anteriormente, os fotoprodutos são marcadamente mais hidrofóbicos comparados à riboflavina, devido à perda do grupamento ribitil das moléculas durante a fotodegradação.

5 Vias de Transdução de Sinal implicadas na função de osteoblastos

Diferentes estágios estão envolvidos no processo de osteoblastogênese incluindo proliferação, síntese de matriz extracelular, amadurecimento e a mineralização. Cada estágio é regulado através da expressão coordenada de fatores de transcrição (Marie et al., 2008). Os mais importantes fatores de transcrição que controlam a formação óssea são Runx2, Osterix, β-catenin, fator de transcrição ativante-4 (ATF4), proteína ativadora-1 (AP-1) e a proteínas de união ao acentuador/CCAAT (C/EBP). Outros fatores de transcrição pertencentes à família *homeo box* (proteínas Msx e Dlx)(Marie et al., 2008), proteínas hélice-volta-hélice (*Id* e *Twist*) (Marie et al., 2008) e o domínio intracelular do receptor Notch (Sciaudone et al., 2003) exercem função no desenvolvimento dos osteoblastos. Além do mais a expressão de todos estes fatores de transcrição é conhecida ser modulada através de vários hormônios (hormônio da paratireóide, estrogênios, glicocorticóides, 1,25-Diidroxivitamina D) (Strewler, 2001) ou fatores de crescimento (BMP, proteína morfogenética; TGFβ, fator de crescimento transformante-β; IGF-1, fator de crescimento semelhante à insulina; FGF, fator de crescimento fibroblástico) (Baylink et al., 1993).

A diferenciação osteoblástica é estimulada através de hormônios e fatores locais, os quais atuam em diversas vias de sinalização dentro das linhagens osteoprogenitoras (Kalajzic et al., 2005; Huang et al., 2007). Várias vias de sinalização tais como BMP (Yamaguchi et al., 2000), fator de crescimento transformante-β, fator de crescimento semelhante à insulina (Niu & Rosen,

2005), fator de crescimento fibroblástico, Wnt (*wingless-type MMTV integration site family*) (Westendorf et al., 2004), Hedgehog (Yamaguchi et al., 2000), Notch (Sciaudone et al., 2003; Zanotti et al., 2008), Fas/FasL (Kovacić et al., 2007), TNF/TNFR (Ding et al., 2009), PI3K/AKT (Ghosh-Choudhury et al., 2002; Kita et al., 2008), integrinas (Takeuchi et al., 1997), caspases (Mogi & Togari, 2003; Miura et al., 2004) e proteínas quinases ativadas por mitógenos (MAPK) (Ge et al., 2007; Xiao et al., 2000) têm sido implicadas (Figura 6). Muitos marcadores são disponíveis e utilizados frequentemente para estudar a influência de vários fatores nas funções e nos estágios de desenvolvimento dos osteoblastos. Certamente, fosfatase alcalina, colágeno tipo I, osteopontina e sialoproteína óssea são marcadores dos eventos iniciais do fenótipo osteoblástico, enquanto a osteocalcina exerce função na mineralização (Stein et al., 1993) (Figura 2).

Osteoblastos regulam a osteoclastogênese e exercem uma função direta e essencial na regulação da função dos osteoclastos dentro do microambiente ósseo. Tem sido demonstrado que o sistema Osteoprotegerina (OPG) /ligante do receptor ativador do NF-κB (RANKL) /RANK exercem função na osteoclastogênese como o mediador final e dominante deste processo (Wright et al., 2009).

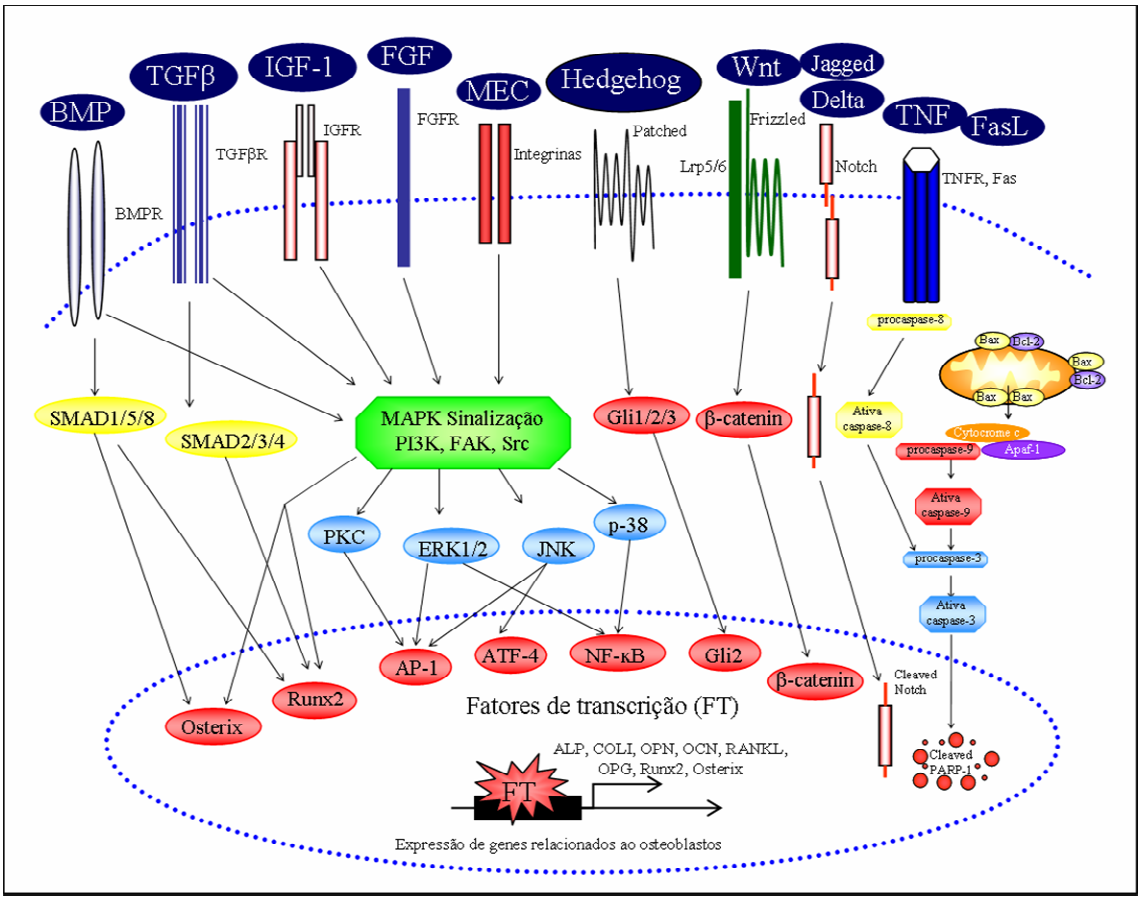

Figura 6. Vias de transdução de Sinal implicadas na regulação das funções do osteoblasto. BMP, proteína morfogenética; TGFβ, fator de crescimento transformante-β; IGF-1, fator de crescimento semelhante à insulina; FGF, fator de crescimento fibroblástico; Wnt, *wingless-type MMTV integration site family*; TNF, fator de necrose tumoral; FasL, ligante Fas; BMPR, receptor de proteína morfogenética; TGFβR, receptor fator de crescimento transformante-β; IGFR, receptor do fator de crescimento semelhante a insulina; FGFR, receptor do fator de crescimento fibroblástico; Lrp5/6, *LDL-related protein 5/6*; SMAD, *mothers against decapentaplegic homologue*; TNFR, receptor do fator de necrose tumoral; Fas, receptor do ligante Fas; MAPK, proteínas quinases ativadas por mitógenos; ERK, quinase reguladas por sinais extracelulares; JNK, *c-Jun N-terminal kinase*; PKC, proteína quinase C; Gli, *glioma-associated oncogene*; Runx2, *runt-related transcription factor-2*; AP-1, proteína ativadora-1; ATF4, fator de transcrição ativante-4; TF, fator de transcrição; COLI, colágeno tipo I; ALP, fosfatase alcalina; OPN, osteopontina; OCN, osteocalcina; BSP, sialoproteína óssea, Bax, *BCL2-associated X protein*; Bcl-2, *B-cell CLL/lymphoma 2*; RANKL, *tumor necrosis factor (ligand) superfamily, member 11*, OPG, osteoprotegerina; PARP-1, *poly(ADP-ribose) polymerase-1*; apaf-1, *apoptotic protease activating factor 1*; caspase, *cysteine-aspartic-acid-proteases*; MEC, matriz extracelular (Adaptado: Trzeciakiewicz et al., 2009).

6 Perfil Quinômico

Proteínas quinases são as chaves regulatórias de importantes processos celulares, incluindo crescimento, resposta ao estresse, diferenciação e apoptose. Aproximadamente 550 quinases têm sido identificadas no genoma humano, exercendo suas funções dentro das células através de uma complexa rede de interações. Para o desenvolvimento de drogas, pesquisas básicas e clínicas envolvendo a análise da transdução do sinal têm investigado profundamente os efeitos de diferentes estímulos em múltiplas proteínas quinases para entender os efeitos de inibidores de quinases, o desenvolvimento de doenças ou os fatores que estimulam o crescimento. Recentemente, a análise do perfil quinoma empregando a tecnologia de arranjos de peptídeos, que compreende sequências de consenso provenientes do quinoma de mamíferos, tem emergido como uma forte ferramenta que gera uma compreensiva descrição das atividades de quinases celulares (Diks et al., 2004; Löwenberg et al., 2005; Tuynman et al., 2007; Parikh et al., 2009). A análise pelo *PepChip Kinomics*^{TM} prove esta ampla abordagem nas alterações das quinases através da análise da atividade de quinases de tecido e células sobre 1024 substratos individuais em um único arranjo. Pequenos volumes de amostras são requeridos para permitir a análise de biópsias, secções e amostras de sangue, como também culturas de células e amostras tumorais.

Amostras lisadas de tecidos ou culturas de células são analisadas em arranjos denominados *PepChip Kinomics*^{TM} contendo 1024 peptídeos em triplicata derivados de sítios de fosforilação conhecidos a partir do sequenciamento de proteínas. Os arranjos são incubados com os lisados celulares ou teciduais. A análise é baseada na detecção do isótopo usando γ-^{33}P-ATP (adenosina trifosfato) marcado radioativamente. Os microarranjos radio-marcados são digitalizados, quantificados e analisados para produção de uma lista de substratos, os quais sofreram mudanças significantes na fosforilação (Figura 7).

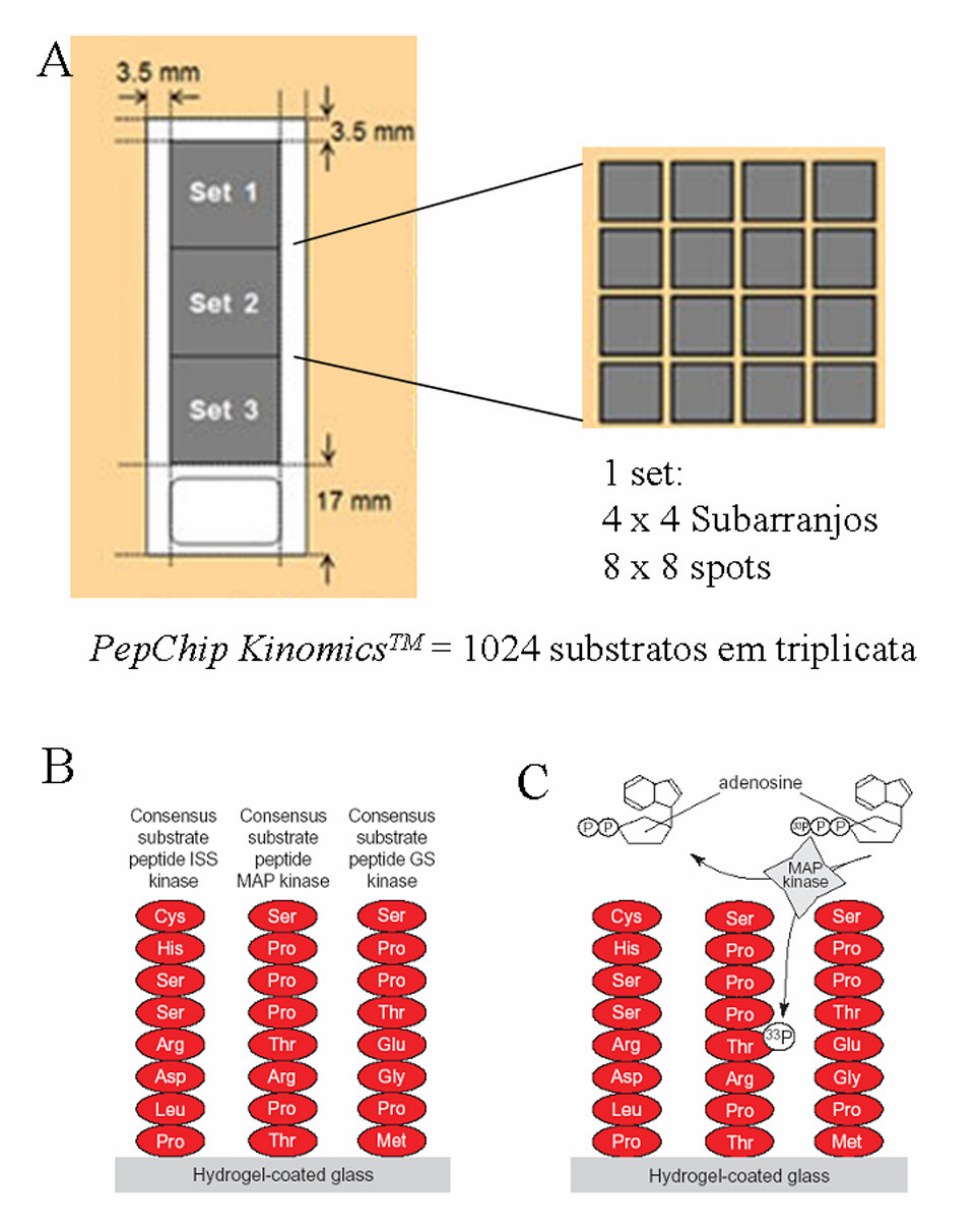

Figura 7. Layout do *PepChip Kinomics*™ . **(A)** Em cada set estão presentes 1024 peptídios, onde os mesmos são numerados de 1-1024; **(B)** Os peptídeos estão ligados ao slide e são constituídos por 8 a 9 aminoácidos, os quais são referentes a sequências de consenso de sítios de fosforilação proveniente de diversas proteínas sequenciadas; **(C)** Proteínas quinases presentes nas amostras de culturas celulares ou biópsias tumorais fosforilam os substratos utilizando γ-^{33}P-ATP (adenosina trifosfato) marcado radioativamente.

Objetivos

Objetivos

O objetivo geral proposto neste projeto foi avaliar os efeitos da riboflavina e riboflavina irradiada na linhagem de pré-osteoblastos estabelecida espontaneamente a partir da calvária de camundongos recém-nascidos (MC3T3-E1).

A caracterização dos efeitos biológicos foi organizada a partir dos seguintes objetivos específicos:

1- Caracterizar através do uso de microarranjos de peptídeos, testes enzimáticos e *western blotting* as vias de transdução de sinal moduladas durante a fase inicial de diferenciação de pré-osteoblastos induzidas por ácido ascórbico e β-glicerofosfato;

2- Análise da citotoxidade da riboflavina e riboflavina irradiada através de testes de viabilidade celular: redução do MTT, incorporação do corante vermelho neutro e análise do conteúdo de ácidos nucléicos;

3- Análise da citotoxidade da riboflavina e riboflavina irradiada na presença indutores clássicos de diferenciação osteoblástica (50µg/mL de ácido ascórbico e 10 mM de β-glicerofosfato) através dos teste de viabilidade celular: redução do MTT, incorporação do corante vermelho neutro e análise do conteúdo de ácidos nucléicos;

4- Efeitos da riboflavina e riboflavina irradiada nos marcadores de diferenciação osteoblástica através da análise da taxa de proliferação, atividade de fosfatase alcalina, síntese e acúmulo de colágeno tipo I e expressão de proteínas ósseas da matriz extracelular (osteocalcina e osteopontina);

5- Análise dos efeitos da riboflavina e riboflavina irradiada na atividade das metaloproteínases MMP-2 e MMP9, as quais são relacionadas com a degradação e remodelamento da matriz extracelular óssea;

6- Identificação de vias de sinalização que foram moduladas através do tratamento com riboflavina e riboflavina irradiada atráves de testes enzimáticos e *western blotting*;

7- Perfil do estado redox celular e do nível de atividade de enzimas oxidantes diante dos tratamentos com riboflavina e riboflavina irradiada.

Capítulo 1

AVALIAÇÃO DAS VIAS DE SINALIAÇÃO NO ESTÁGIO INICIAL DE DIFERENCIAÇÃO OSTEOBLÁSTICA INDUZIDA POR ÁCIDO ASCÓRBICO E β-GLIGEROFOSFATO

“SCREENING OF SIGNALING PATHWAYS IN THE INITIAL-STAGE OF ASCORBIC ACID/β-GLYCEROPHOSPHATE-INDUCED OSTEOBLASTIC DIFFERENTIATION”

SCREENING OF SIGNALING PATHWAYS IN THE INITIAL-STAGE OF ASCORBIC ACID/B-GLYCEROPHOSPHATE-INDUCED OSTEOBLASTIC DIFFERENTIATION

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ABSTRACT Despite a profusion of reports about the biological properties of ascorbic acid such as an inductor of osteoblast differentiation, not much information about the molecular aspects of this activity is available in the scientific literature. Therefore, we decided to identify the signaling pathways modulated by ascorbic acid and β-glycerophosphate (AA/β-GP) responsible for the initial stage of osteoblast differentiation. For this purpose we combined a peptide array, exhibiting specific consensus sequences (potential substrates), for protein kinases and traditional biochemical techniques. Unlike other differentiation inductors, preosteoblast cells cultured for 7 days in the presence of AA/β-GP showed downregulation of PI3K and Src kinase cascades and activation of mediators of apoptosis. Kinomic profiles of AA/β-GP-induced osteoblast differentiation process revealed 27 different kinase substrates with either significantly increased or decreased phosphorylation. We observed a significant decrease in PI3K-associated signal transduction, including reduced phosphorylation of substrates for Akt and PKC; in agreement, a concomitant increase in phosphorylation of a substrate for GSK3β was apparent after the initial phase of differentiation. We also found that substrates for Syk and PAK2 showed considerable reduction in phosphorylation, while an increase in phosphorylation of substrates for PKA and FAK were significant. Another important finding was the induction of osteoprotegerin by AA/β-GP, which is a principal negative modulator of osteoclast development. Our study brings out new molecular aspect of the osteogenic property of AA/β-GP. Besides, these results reinforce the applicability of peptide arrays in providing molecular information about different biological processes.

KEYWORDS: ASCORBIC ACID; B-GLYCEROPHOSPHATE; OSTEOBLAST DIFFERENTIATION; CASPASE; PI3K; MAPK

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Abbreviations Used: PI3KR1, phosphoinositide-3-kinase, regulatory subunit 1 (alpha); Akt1, v-Akt murine thymoma viral oncogene homolog 1; RPS6KA2, ribosomal protein S6 kinase, 90kDa, polypeptide 2; RPS6K, Ribosomal protein S6 kinase; GSK3β, glycogen synthase kinase 3 beta; PAK2, p21-activated protein kinase 2; PAK1, p21-activated protein kinase 1; ATM, Ataxia-telangiectasia mutated; DNPK1; DNA-dependent protein kinase catalytic subunit; PRKDC, protein kinase, DNA-activated, catalytic polypeptide; EGFR, Epidermal growth factor receptor; p38, p38 kinase; ERK1/2, Extracellular signal-regulated kinase 1/2; FAK, Focal adhesion kinase; SAPK/JNK, stress-activated protein kinase/Jun-amino-terminal kinase; PKC, Protein kinase C; PKA, Protein kinase, cAMP-dependent; PKCμ, protein kinase D1; Src, v-Src avian sarcoma (Schmidt-Ruppin a-2) viral oncogene; SYK, spleen tyrosine kinase; MAPK1, mitogen-activated protein kinase 1; INSR, insulin receptor; CHKβ, choline kinase beta; CHKA, choline kinase alpha; ADRBK2, adrenergic, beta, receptor kinase 2; PRKG1, protein kinase, cGMP-dependent, type I; PDGFRB, platelet-derived growth factor receptor, beta polypeptide; VEGFR-1, soluble vascular endothelial growth factor receptor-1; BCR, breakpoint cluster region; BTK, Bruton agammaglobulinemia tyrosine kinase; ABL1, c-abl oncogene 1, receptor tyrosine kinase; JAK2, Janus kinase 2; MAP3K10, mitogen-activated protein kinase kinase kinase 10; CSNK2A1, casein kinase 2, alpha 1 polypeptide; SGK1, serum/glucocorticoid regulated kinase 1; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); DDR1, discoidin domain receptor tyrosine kinase 1; PKA, protein kinase, cAMP-dependent, catalytic, alpha; ATF2, activating transcription factor 2; ELK1, member of ETS oncogene family; RUNX2, runt-related transcription factor 2; SP7 or Osterix, Sp7 transcription factor; BAX, BCL2-associated X protein; BCL-2, B-cell CLL/lymphoma 2; RANKL, tumor necrosis factor (ligand) superfamily, member 11, OPG, osteoprotegerin; PARP-1, poly(ADP-ribose) polymerase-1; PTEN, phosphatase and tensin homolog; PP2A, protein serine/threonine phosphatase; SHP-2 or Ptpn11, protein tyrosine phosphatase, non-receptor type 11.

INTRODUCTION

Mineralization is a fundamental step towards osteoblast differentiation, in which mineral particles are deposited into a collagen matrix. In MC3T3-E1 preosteoblasts, an osteoblastic cell line derived from normal mouse calvaria, differentiation is determined by expression of osteoblast-like differential markers and the formation of mineralized extracellular matrix. Exposure to L-ascorbic acid (reduced vitamin C, AA) and β-glycerophosphate (β-GP) have been shown to support this process by distinct mechanisms (Chung et al., 1992, Franceschi, 1992; Franceschi et al., 1994). Chung et al. have determined that β-GP favors mineralization through an increase in phosphate ion availability, whereas AA has an established role as a reductant for the iron prostethic group of hydroxylase enzymes responsible for collagen biosynthesis. Thus, AA/β-GP stimulate procollagen hydroxylation, processing, and fibril assembly followed by the dramatic induction of specific genes associated with the osteoblast phenotype, including alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN) and the PTH/PTH-related protein receptor (Quarles et al., 1992; Franceschi and Iyer, 1992). Despite several reports in the literature addressing the differentiation of MC3T3-E1 preosteoblasts under different aspects, there is little information about molecular mediators involved in the signal transduction culminating in the osteoblast phenotype. Although multiple biological functions have been identified for AA/β-GP, the precise molecular mechanisms underlying these actions have not yet been fully elucidated. In this context, protein kinases activated along the distinguishable stages can play a crucial role to determine the final fate of cell differentiation and the quality of newly formed bone.

Although, the capacity of AA/β-GP to stimulate the differentiation of preosteoblasts is very well established, the molecular mechanisms involved in this process still need to be elucidated in details. The activities of any differentially expressed proteins have often remained undefined due to incomplete characterization of relevant post-translational modifications which may act to modulate protein activity. Reversible phosphorylation catalyzed by kinases is probably the most important regulatory mechanism in eukaryotes. Protein kinases play a crucial role in a variety of cellular processes including cytoskeletal reorganization, cell cycle progression, cell survival and differentiation. Therefore, we decided to investigate the kinomic profiling of osteoblasts cultured, for 7 days, in the presence of AA/β-GP by using peptide arrays (PepChip).

In the present study, a kinomic profiling at initial phase of AA/β-GP-induced osteoblastic differentiation was obtained by using a peptide array (PepChip) containing 1,024 different kinase-specific consensus sequences, generating a comprehensive overview of cellular kinase activity. The method, as first described by Diks et al. (2004), allows a detection of signaling pathways in cell lysates.

In summary, the kinomic profile revealed suppression of proliferation and survival pathways (PI3K/Akt and Src/ERK) and activation of proapoptoic effectors (caspase-8, caspase-9 and caspase-3) at initial phase of osteoblastic differentiation in preosteoblast cells induced by ascorbic AA/β-GP.

MATERIALS & METHODS

MATERIALS

Ascorbic acid and β-glycerophosphate were purchased from Sigma. MC3T3-E1 preosteoblast cells were from American Type Culture Collection (ATCC, Rockville, MD).

Polyclonal antibodies against phospho-p38 Thr180/Tyr182, phospho-p42/44 (ERK1/2), phospho-SAPK/JNK Thr183/Tyr185, phospho-(Tyr)-PI3K p85,

phospho-Akt Thr308, phospho-p70 S6 kinase Thr389, phospho-p90RSK Ser380, phospho-Src Y416, phospho-Syk (Tyr525/526), phospho-PKC (pan) Ser660, phospho-PKD/PKCμ Ser744/748, phospho-GSK3β Ser9, phospho-PTEN Ser380/Thr382/383, phospho-PP2A Tyr 307, phospho-SHP-2 Tyr542, phospho-Elk-1 Ser383, phospho-ATF-2 Thr71, phospho-PAK2 Ser20, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PAKα, Bcl2, Bax, OPG, RANKL and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP-1 (p116/p25), phospho-FAK Tyr397, β-catenin were from Upstate Biotechnology, Inc. (Lake Placid, NY).

Caspases 3, 8 and 9 Colorimetric Assay Kits were obtained from R&D Systems (Minneapolis, MN). Kinase array slides (PepChip™) were purchased from Pepscan Systems (The Netherlands). γ-^{33}P-ATP (adenosine triphosphate) was purchased from Amersham Biosciences.

CELL CULTURE CONDITION

MC3T3-E1 preosteoblast cells were routinely grown in modified alpha minimum essential medium (α-MEM) without AA, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics and incubated in a humidified atmosphere at 37 °C and at 5% CO_2. For all experiments, cells were plated at an initial density of 50,000 cells/cm^2. All treatment conditions were performed after 24h of cell attachment (day 0). Differentiation and mineralization were induced by adding osteogenic medium (AA/β-GP) containing 50 μg/ml AA and 10 mM β-GP for 7 days. After plating, cells were treated every three or four days. Cells maintained with medium alone were used as a control (Ctrl) for all experiments.

mRNA EXPRESSION OF MATRIX PROTEINS AND OSTEOBLAST MARKERS

Total RNA was isolated from cells using Trizol reagent (Sigma-Aldrich Zwijndrecht, Netherlands) according to manufacturer's instructions. Reverse transcription was performed on 5 ng of total RNA using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 μl. Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Invitrogen, Breda, Netherlands) on the Biometra PCR system. PCR primers for mice were selected from multiple exons: alkaline phosphatase (NM_007431), 5′-aacccagacacaagcattcc-3′ and 3′-cgaagggtcagtcaggttgt-5′; type I collagen (NM_007742.2), 5′-gagcggagagtactggatcg-3′and 3′-gttcgggctgatgtaccagt-5′; osteopontin (NM_009263.1), 5′-tctgatgagaccgtcactgc-3′ and 3′-cacccgagagtgtggaaagt-5′; osteocalcin (NM_007541.2), 5′-gcgctctgtctctctgacct-3′ and 3′-gccggagtctgttcactacc-5′; Runx2 (NM_009820), 5′-cccagccacctttacctaca-3′ and 3′-tatggagtgctgctggtctg-5′; Osterix (NM_130458), 5′-actggctaggtggtggtcag-3′ and 3′-ggtagggagctgggttaagg-5′; β-actin (NM_007393), 5′-tctcttccagccctctttca-3′ and 3′-atggtggtgcctccagatag-5′. The cycling program was 94°C for 2 min, 58°C for 60 seconds, 72°C for 60 seconds for the first cycle and 94°C for 30 sec, 58°C for 60 seconds, 72°C for 60 seconds for 30 cycles. PCR products were analyzed by 3% agarose gel electrophoresis and stained with ethidium bromide. The level of mRNA expression is expressed relative to the β-actin level. All experiments were performed in triplicate.

DETERMINATION OF CELL CYCLE PROGRESSION AND APOPTOTIC CELL DEATH WITH FLUORESCENCE-ACTIVATED CELL SORTER (FACS)

MC3T3-E1 cells were plated on 6 cm diameter dishes at a density of 50,000 cells/cm^2 and cultured in the presence of 10% FBS medium for 24 h. For serum deprivation experiments cells were washed three times in PBS and cultured in serum free αMEM. After 24 h of serum starvation, cells were treated with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate for 24 h. After this period the cells were harvested and deattached by trypsinization. Cells were washed with cold PBS and resuspended in paraformaldehyde 2% in PBS at 4 °C. Subsequently, 1 ml of propidium iodide solution was added and maintained at 4ºC for 15 min. The analysis was performed in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA,USA). It was analyzed 10,000 events per sample. DNA content was evaluated using a FL2 detector in a linear scale. To eliminate cell aggregates, the cell population to be analyzed was selected from a bivariate histogram showing the area (FL2A) *versus* the width (FL2W) of the signal FL2. The analysis of cell percentage in the different phases of the cell cycle (Sub-G_1, G_0/G_1, S/M) was performed using the CellQuest 3.4 software (BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate and the results shown in the table represent the means ± standard errors. Statistical significance was analyzed by Student's t-test. Differences were considered significant when the *p* value was less than 0.05.

KINOME ARRAY ANALYSIS

A kinase substrate peptide array consisting of 1,024 peptides with specific phosphorylation sites was used to comprehensively evaluate the kinome during the initial phase of osteoblastic differentiation, as has been previously described (Diks et al., 2004, Löwenberg et al., 2005; Tuynman et al., 2008; Parikh et al., 2009). After AA/β-GP treatment for 7 days, the cells were washed in ice-cold PBS and harvested in lysis buffer (Pierce Biotechnology, Inc.) supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations were determined using Bradford analysis (Bio-Rad). The protocol of the kinome array is described in detail on the website (http://www.pepscanpresto.com/files/PepChip%20Kinase%20Lysate%20Protocol_v5.pdf). Stimulations were terminated by an ice-cold phosphate-buffered saline wash and the cells were lysed as described by Diks et al. (2004). Experiments were performed in triplicate and repeated independently.

DATA ACQUISITION AND STATISTICAL ANALYSIS OF PEPCHIP™ ARRAY

After drying, the glass slides were exposed to a phosphor imager plate for 72 hours. Acquisition of the peptide array was performed using a phospho-imager (Storm™, Amersham Biosciences, Sweden). The level of incorporated radioactivity, which corresponds to the phosphorylation status was quantified by array software (EisenLab ScanAlyze, version 2.50) and as described elsewhere (Diks et al., 2004, Löwenberg et al., 2005; Tuynman et al., 2008). Datasets from chips were then submitted to statistical analysis. Basically, spot replications were scrutinized for consistency using two indexes: one of them is the ratio standard deviation / average (SD/A) and the other is the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to indexes are SD/A < 20% and 80% < A/M < 120%. Standard statistical analysis follows with calculated fold changes and t-tests for assessing significantly different averages between chips.

WESTERN BLOTTING ANALYSIS

Following treatment of cells for 7 days, the medium was aspirated and the cells were washed with cold physiological solution. Cells were then incubated

in 200 µl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using Lowry method (1951). An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 75 µg of protein, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 ºC with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence ECL. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to β-actin ratio). The graph represents the means ± standard errors. Statistical significance was analyzed by Student's t-test. Differences were considered significant when the *p* value was less than 0.05.

CASPASES 3, 8 AND 9 ACTIVITY ASSAYS

Caspase activities were determined by the measurement at 405 nm of p-nitroaniline (pNA) released from the cleavage of Ac-DEVD-pNA, IETD-pNA and LEHD-pNA as substrates of caspases 3, 8 and 9, respectively. Enzyme activities were expressed in pmol/min and the extinction coefficient of pNA was 10,000 $M^{-1}cm^{-1}$. Graph represents the means ± standard errors. Statistical significance was analyzed by Student's t-test. Differences were considered significant when the *p* value was less than 0.05.

RESULTS

KINOME PROFILING OF AA/β-GP-INDUCED OSTEOBLASTIC DIFFERENTIATION

Peptide arrays exhibiting specific consensus sequences (potential substrates) for protein kinases were used to provide a global analysis of cellular kinase activity in osteoblasts. *In vitro* phosphorylation of peptide arrays by cell lysates showed that osteoblasts contain substantial kinase activity; since almost all substrate peptides incorporated [γ-^{33}P]ATP. The data reported here are, to our knowledge, the first kinomic profiling of initial stage differentiation of a preosteoblastic cell line.

Since almost all cellular biochemical pathways are under strict control by reversible phosphorylation of rate-limiting enzymes, we assumed that the effects of AA/β-GP-induced osteoblastic differentiation would be reflected in altered activity of cellular kinases during the initial phase of process. *In vitro* phosphorylation of peptide arrays after 7 days of culture revealed substantial changes in kinase activity. Analysis of kinomic profiles of AA/β-GP-induced osteoblastic differentiation process revealed 27 different kinase substrates with either significantly increased or decreased phosphorylation (Table 1). We found a significant decrease in PI3K-associated signal transduction, including reduced phosphorylation of substrates for Akt and PKC. In agreement, a concomitant increase in phosphorylation of a substrate

Table **1.** Peptide substrates with significantly altered phosphorylation by lysates from preosteoblastic cells treated with AA/β-GP for 7 days

FC	PEP_NR	Sequence	PH-SITE	Kinase
2,71	779	DSTNEYMDMKP	Y721	CHKB, CHKA
2,32	920	GKRHRYRVLSS	Y80	PKA
2,12	733	RKMKDTDSEEE	T79	INSR, EGFR, FAK
2,00	394	GKKATQASQEY	S140	ATM, PRKDC, DNPK1
1,76	344	RRGDSYDLKDF	S440	PKA
1,71	891	SPPRSSLRRSS	S36	GSK3B
1,60	523	SDDVRYVNAFK	Y1213	VEGFR-1
1,53	659	IGEGTYGVVYK	Y15	PK161
1,40	406	ALDFRTPRNAK	T161	ADRBK2
1,21	771	RKGHEYTNIKY	Y546	PDGFRB
0,82	676	LTRIPSAKKYK	S104	PKC
0,76	910	SSDDDYDDVDI	Y381	SYK
0,76	68	AEKPFYVNVEF	Y177	BCR
0,74	416	KVVALYDYMPM	Y222	BTK, ABL
0,72	556	LMDNAYFCEAD	Y534	JAK2
0,67	805	YVEKFSYKSIT	S415	PKC
0,65	332	MNEVTYSTLNF	T492	MAP3K10
0,64	356	KKRKRSRWNQD	S20	PRKG1
0,62	304	RGQRDSSYYWE	S338	PAK1, PAK2
0,61	183	YGGLTSPGLSY	T431	MAPK1
0,59	576	VEDNRSQVETD	S285	CK2
0,55	885	RDRSSSAPNVH	S363	AKT, SGK
0,53	185	GTRRGSPLLIG	S205	PKC
0,51	961	AENPEYLGLDV	Y1248	ERBB2
0,50	752	FFPFHSPSRLF	S19	CDK5
0,49	364	TEDQYSLVEDD	Y607	PIK3R1
0,47	459	LSNPAYRLLLA	Y513	DDR1

NOTE: The fold induction of each substrate, their corresponding protein kinases, and chip peptide number are provided.
Abbreviation: FC, fold change

for GSK3β occurred apparently after the initial phase of differentiation. We also found that substrates for Syk and PAK2 showed considerable reduction in phosphorylation, while an increase in phosphorylation of PKA and FAK substrates were significant (Fig. 1).

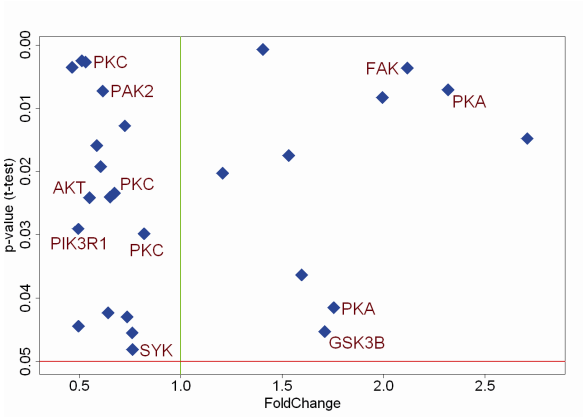

Figure 1. Scatter plots of kinomic analysis to AA/β-GP-induced osteoblastic differentiation. The X-axis represents the relative fold change value relative at the changes of kinases activity between the controle group and AA/β-GP-induced osteoblastic differentiation group. The Y-axis is the significance of the change by t-test P-value. The line represents the kinase up and downregulated.

Table 2. Evaluation of cell cycle changes in preosteoblastic MC3T3-E1 after treatment with osteogenic medium

Cells	Sub-G_1	G_0/G_1 phase	Synthesis phase/Mitosis
Control	1.36 ± 0,30	39.60 ± 1.46	59.05 ± 1.17
AA/β-GP	1.67 ± 0.66	48.56 ± 1.31*	49,785±1,96*

After 24 h of serum starvation cells were treated with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate for 24 hours. Cells were stained with propidium iodide and the DNA content was analyzed by flow cytometry. Values of the percentages of MC3T3-E1 remaining in the Sub-G_1, G_0/G_1 phase and Synthesis/Mitosis phase are shown on table. Results represent the means ± SEM of three independet experiments. * $P<0.05$ *vs.* Control.

VALIDATION OF KINOME PROFILE RESULTS

The results of peptide array profiling indicate that AA/β-GP decreased PI3K and Akt activity. To confirm these observations, phosphorylation levels of downstream kinases in the PI3K signaling pathway were evaluated by Western blot using phosphospecific antibodies (Fig. 2A). In agreement with the peptide array analysis, phosphorylation levels of PI3K, Akt, GSK3β, p70 S6 Kinase, p90RSK, PKC Pan and PKD/PKCµ were significantly diminished as revealed by western blot. The increase in GSK3β activity, an

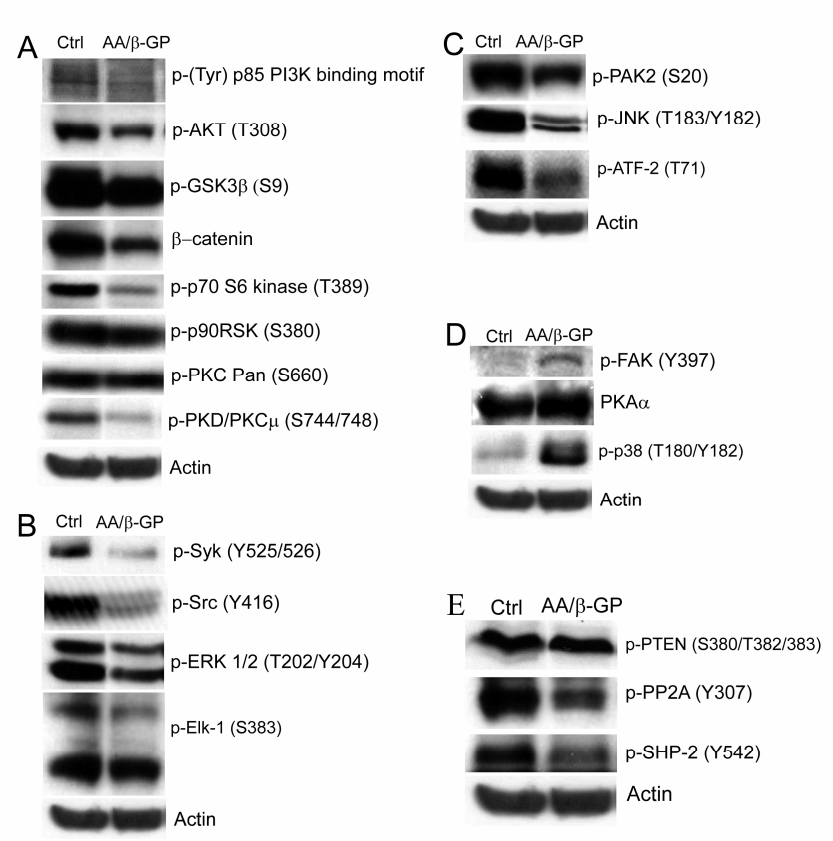

Figure 2. Validation of kinome profile results for kinases involved in initial-stage of ascorbic acid-induced osteoblastic differentiation. Western blot analysis confirms kinomic analysis and identifies PI3K, AKT, GSK3β, PKC, Syk, PKA, FAK, PAK2 as a target to modulation by AA/β-GP. (A) Downregulation of PI3K/AKT pathway with a markedly reduced phosphorylation in the p70 S6 kinase, p90RSK, PKC Pan, PKD/PKCμ, while the increase GSK3β activity can see observed by decrease of active β-catenin. (B) Downregulation of Syk and Src/ERK pathways. (C) Reduced phosphorylation and activity of PAK2, SAPK/JNK. (D) Increase of FAK and P38 activity and increase of PKA expression. (E) Increase of PP2A activity and decrease of SHP2 activity. The phosphorylation and expression status of proteins in whole-cell lysates was assayed using phosphospecific mAbs on Western blot. Anti-actin was used as a control.

event associated with decreased phosphorylation of Ser9 by Akt, can be confirmed by a reduction in active β-catenin, a GSK3β substrate involved with Wnt signaling.

Important regulatory kinases involved in other pathways, such as Syk, Src and ERK 1/2, were downregulated, as shown by their phosphorylation status (Fig. 2B). The reduction of PAK2 activity, as detected by the peptide arrays, was confirmed via observation of a decrease in autophosphorylation at the Ser20 residue. The activity of PAK2 and Syk has been shown to positively regulate the cellular response to stress by activation of SAPK/JNK. The decrease in phosphoryation of SAPK/JNK can be reflected through the suppression of both PAK2 and Syk (Fig. 2C). In addition, FAK and PKA were also consistently up-modulated in the peptide array (Fig 2D). Importantly, the AA/β-GP stimulated PP2A and suppressed SHP-2 activities, which indicates that these enzymes might be a target for the osteoblastic differentiation process. Moreover, PTEN, the main regulator of PI3K signal transduction, did not show a change in activity (Fig. 2E).

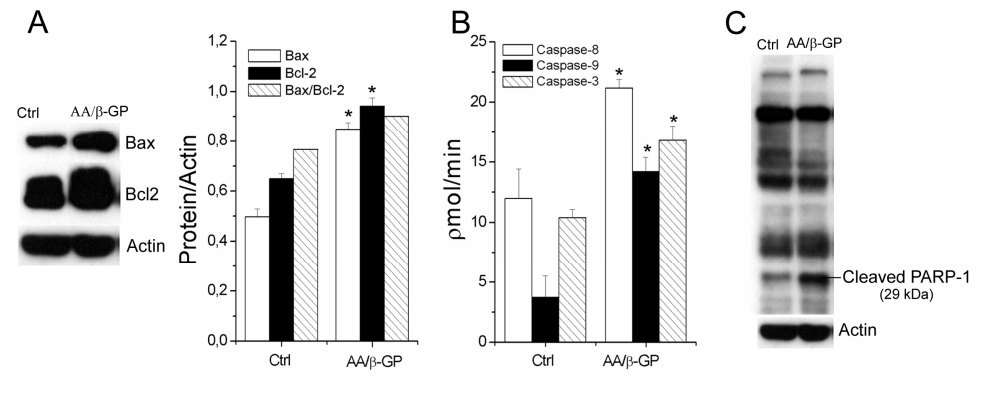

Figure 3. AA/β-GP-induced osteoblastic differentiation mediates changes in key apoptotics regulators. MC3T3-E1 cells were treated for 7 days with 50 µg/ml of ascorbic acid and 10 mM β-glycerophosphate. (A) Western blotting analysis of Bcl-2 and Bax levels and quantitative analysis of Bax/Bcl-2. Quantification of immunoband intensities from Western blotting analysis was determined by densitometric scannig. Data were expressed as Bax/Bcl-2 ratio. (B) Colorimetric assay was performed to determine the activation of caspase-8, caspase-9 and caspase-3. (C) Western blot analysis of cleaved PARP-1. Results represent the means ± SEM of three independet experiments. * *P*<0.05 *vs.* Control. Anti-actin was used as control.

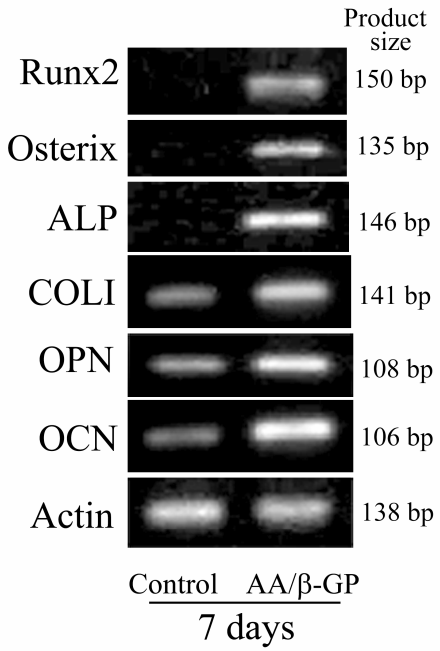

Figure 4. Analysis of key differentiation markers in MC3T3-E1 preosteoblast cells after treatment with AA/β-GP. After culturing the cells in the absence or presence of osteogenic medium the mRNA expression of ALP, COL I, and the osteoblast markers Runx2, Osterix, OPN and OCN were analyzed by RT-PCR.

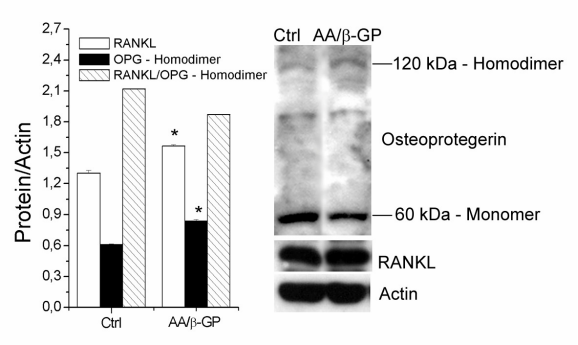

Figure 5. Expression of RANKL, OPG and RANKL/OPG-Homodimer. MC3T3-E1 cells were treated for day 7 with 50 µg/ml of ascorbic acid and 10 mM β-glycerophosphate. RANKL and OPG-Homodimer were upregulated in osteoblasts after treatment according to Western blot analysis. Results represent the means ± SEM of three independet experiments. * P<0.05 vs. Control. Anti-actin was used as control.

AA/β-GP-INDUCED OSTEOBLASTIC DIFFERENTIATION CAUSES INCREASE OF BAX/BCL-2 RATIO, CASPASE-9, CASPASE-8 AND CASPASE-3 ACTIVATION AND SUBSEQUENT CLEAVAGE OF PARP IN OSTEOBLASTS

After 7 days of treatment, we observed an enhancement of Bax/Bcl-2 ratio by nearly 1.2-fold ($P<0.05$; Fig. 3A). Besides, AA/β-GP caused a significant activation of caspase-8, caspase-9 and caspase-3 (Fig. 3B) and cleavage of PARP-1 (Fig. 3C). To verify whether AA/β-GP could trigger apoptotic cell death, we monitored cell proliferation and conducted both FACS cell-cycle progression and apoptosis analysis. We found that AA/β-GP caused G_0/G_1 arrest in MC3T3-E1 cell after 24h (control *versus* AA/β-GP: 39.60 *versus* 48.56), while few cells were found in hypodiploid sub-G_1 apoptotic peak. (1.36 *versus* 1.67) (Table 2). Despite caspase activation, apoptosis is not the dominant response towards AA/β-GP, once markers of differentiation such as extracellular matrix proteins (type I collagen, osteopontin and osteocalcin), transcription factors (Runx2 and Osterix) and alkaline phosphatase were highly expressed in the presence of osteogenic medium (Fig. 4).

AA/β-GP-INDUCED OSTEOBLASTIC DIFFERENTIATION DOWNREGULATES THE RATIO OF RANKL/OPG

Figure 5 shows that AA/β-GP significantly increased the production of OPG-Homodimer and RANKL, however we observed a decrease of the RANKL/OPG ratio. These findings suggest that the effects of AA/β-GP appear also to be mediated, at least in part, by an increase in OPG-Homodimer.

DISCUSSION

Gene array technologies have allowed researchers to define the transcriptome of MC3T3-E1 pre-osteoblastic cells treated with ascorbic acid (Carinci et al., 2005) and ascorbic acid plus β-glycerophosphate (Beck et al., 2001). However, many aspects of differentiation along the osteogenic lineage require complex genetic and biochemical regulatory mechanisms (Lian and Stein, 2003; Harada and Rodan, 2003). Therefore, experimental procedures providing functional/activity status of a set of proteins is important to give more insights about a specific physiological process. In this context, we employed PepChip array technique in order to obtain a kinomic profiling of preosteoblastic cells cultured in the presence of AA/β-GP. To provide a signaling pathway overview of osteoblastic differentiation triggered by AA/β-GP, we confirmed the differentiation status of cells cultured with AA/β-GP for 7 days. In agreement with other works (Quarles et al., 1992; Franceschi and Iyer, 1992; Wang et al., 1999) we detected an increase in the expression of specific markers: COLI, ALP, RUNX2, Osterix, OCN and OPN.

In this study we observed that AA/β-GP modulate different kinases as well as caspases. We found that the combination of AA plus β-GP suppresses PI3K/Akt and Src/ERK 1/2 signaling cascades, which indicates that downregulation of both cascades is essential for the initial phase of osteoblastic differentiation. In accordance to our findings, the inhibition of PI3K pathway has been related to the differentiation process of B16 melanoma (Buscà et al., 1996), human promyelocytic HL60 leukemia cell line (Peiretti et al., 2001), myogenic cells line (C2C12) (Viñas et al., 2002) besides acceleration of chondrocyte terminal

differentiation (Kita et al., 2008). On the other hand, PI3K/Akt is one of the key players in the signaling of potent bone anabolic factors. For instance, growth factors like PTH and IGF-1 can activate the PI3K pathway, followed by the activation of Akt (Yamamoto et al., 2007; Nakasaki et al., 2008), while Wnt proteins prolong the survival of osteoblasts and uncommitted osteoblast progenitors via activation of Src/ERK and PI3K/Akt signaling cascades (Almeida et al., 2005). Our group has reported a decrease in Src activity during the ascorbic acid-induced differentiation of preosteoblast cells (Zambuzzi et al., 2008). In addition, we also reported a temporal regulation of PI3K/Akt pathway and ERK 1/2 MAPK during ascorbate-induced osteoblast differentiation in MC3T3-E1 (Zambuzzi et al. 2008), however, it is important to mention that in this work we have not include a condition without AA/GP.

In addition, we also found an expressive negative modulation of PAK2 and Syk kinases. p21-activated protein kinase (PAK) was first identified as Rac/Cdc42 GTPases activated kinases (Manser et al., 1994) and can be activated in response to hyperosmolarity, irradiation, UV light, and DNA-damaging chemotherapeutic drugs such as cytosine β-$_D$-arabinofuranoside and cisplatin. Syk is a nonreceptor type of protein tyrosine kinase ubiquitously expressed by hematopoietic cells and is also expressed in epithelial, endothelial (Kurosaki et al., 2000; Inatome et al., 2001) and osteoblastic cells (Rezzonico et al., 2002). Recent studies suggest that PAK2 and Syk kinases act as upstream regulators of JNK and p38 MAPK pathways (Frost et al., 1996; Zhang et al., 1995). Miah et al. (2004) demonstrate that PAK2 and Syk positively cooperate to regulate cellular responses to stress by activation of JNK under hyperosmotic conditions. Syk is a major upstream effector of PI3K/Akt pathway and participates in PI3K activation, which plays a significant role in regulation of cancer cell motility (Jiang et al., 2002; Mahabeleshwar and Kundu, 2003). It has been suggested that Syk plays a critical role in cell morphogenesis, growth, migration, and survival (Inatome et al., 2001). Therefore, the effects of AA/β-GP in the downregulation of PI3K and JNK could be associated with decreased activity of Syk and PAK2.

In relation to PKA, we noticed an increase of activity and expression of this kinase. Wu et al. (2007) reported that the increase of AA uptake and consequent promotion of differentiation in preosteoblast cells MC3T3-E1 is associated with phosphorylation of sodium-dependent vitamin C transporter 2 (SVCT2) by PKA.

Another point explored in this study was the expression/activity of potential apoptotic effectors in cell differentiation. Despite displaying growth arrest, activation of caspase-8, caspase-9 and caspase-3, an increase of Bax/Bcl-2 and cleavage PARP-1, apoptosis was not the dominant process triggered by AA/β-GP. Activation of caspases are required for BMP-4 osteoblastic differentiation in MC3T3-E1 (Mogi and Togari, 2003), while Miura et al. (2004) demonstrated that caspase-3 is crucial for the differentiation of bone marrow stromal stem cells by influencing TGF-β/Smad2 pathway and cell cycle progression. On the other hand, androgens stimulates osteoblast and osteocyte apoptosis through an increase of Bax/Bcl-2 ratio even in anabolic settings and suggest that enhanced apoptosis can be associated with anabolic stimulation of new bone growth (Wiren et al., 2006). In relation to PARP, Harnacke et al. (2005) showed that the down-modulation of PARP-1 in human TUR leukemia cells restores transcriptional responsiveness for differentiation and cell cycle arrest.

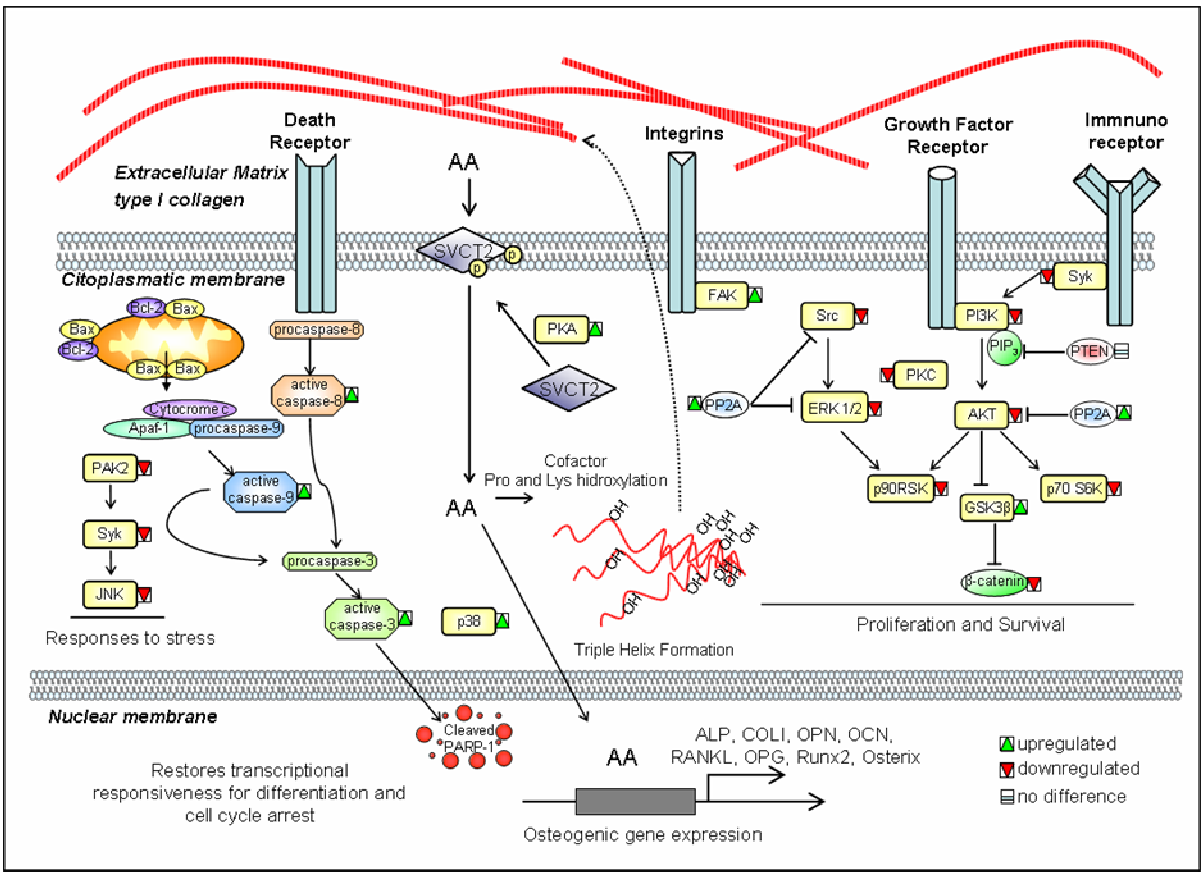

Figure 6. Schematic representation of the molecular mechanism of AA/β-GP-induced differentiation of preosteoblast. Data presented in the report revealed that AA/β-GP exhibits multi-activities, which culminate with the initial phase of differentiation process. AA/β-GP caused de suppression of two important signaling cascade pathway involved in cell survival/proliferation (PI3K/AKT and Src/ERK 1/2). Importantly, our data also show that AA/β-GP-induced differentiation was accompanied by increase of Bax/Bcl-2 ratio, activation of caspase-8, caspase-9, caspase-3 and cleaved PARP-1, while PAK2 and JNK/SAPK, kinases involved in apoptotic signaling events decrease the activity during the differentiation process. Down-modulation of PARP-1 has been associated with restore transcriptional responsiveness for differentiation and cell cycle arrest. The phosphorylation of Sodium-dependent vitamin C transporter (SVCT) 2 by PKA results in an increase of AA uptake and and consequent promotion of osteoblast-like differentiation in MC3T3-E1 cells. Impotantly, the AA/β-GP treatment modulated the activity levels of same phosphatases by stimulation of PP2A and suppression of SHP-2, which indicates that this enzymes can be a target for osteoblastic differentiation.

The role of AA/β-GP in collagen matrix production and expression of osteoblastic differentiation markers has been characterized. However the implication in the OPG and RANKL expression is not clear. OPG is produced by osteoblasts and other cell types (Simonet et al., 1997). Extrinsic or intrinsic perturbations of OPG/RANKL balance will directly result in the imbalance of bone remodeling leading to skeletal diseases such as osteoporosis (Horwood et al., 1998; Nagai and Sato, 1999). It has been suggested that the ratio between OPG and RANKL expression levels in osteoblastic cells is a key factor in bone resorption. We have observed the reduction of the RANKL/OPG ratio at the initial phase of osteoblastic differentiation, which mostly reflected an increase in OPG-Homodimer proteins levels that leads to an increase in the affinity for RANKL (Theoleyre et al., 2004). Therefore, AA/β-GP-induced

osteoblastic differentiation decreases osteoclastogenesis and promotes the OPG-Homodimer formation that is required for inhibition of the RANKL/RANK receptor interaction.

CONCLUSION

Our findings brought out new information to the literature in relation to the biological action of AA/β-GP in the osteogenic process and also pointed out some molecular differences of their action in relation to other osteogenic inductors. A schema summarizing the results is presented in Fig 6.

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Capítulo 2

SUPERÓXIDO DISMUTASE, CATALASE E GLUTATIONA PEROXIDASE APRESENTAM BAIXA ATIVIDADE EM OSTEOBLASTOS DIFERENCIADOS

“SUPEROXIDE DISMUTASE, CATALASE AND GLUTATHIONE PEROXIDASE DISPLAY LOW ACTIVITIES IN DIFFERENTIATED OSTEOBLASTS”

Superoxide dismutase, catalase and glutathione peroxidase display low activities in differentiated osteoblasts

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ABSTRACT Reactive oxygen species play important role in normal as well as pathological conditions. In this study, the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST) and the levels of total GSH and malondialdehyde (MDA) were evaluated after treatment of MC3T3-E1 cells with osteogenic inductors ascorbic acid and β-glycerophoshate. We observed that at the initial stage of osteoblast differentiation, CAT, total SOD and GPX presented lower activities. However, the expression of Mn-SOD was higher in the differentiated cells. Total GSH and MDA levels remained unchanged. Our findings indicated that the variation on the efficiency of antioxidant enzymes might guarantee low level of ROS, which is able to regulate signal transduction pathways, without affecting the osteoblasts functionality. On the other hand, the osteoblastic differentiation state increases the sensitivity of these cells towards the oxidative damage which can be relevant to management of bone diseases.

Keywords: ascorbic acid, β-glycerophosphate, osteoblast differentiation, antioxidant enzymes, MDA, GSH, catalase, superoxide dismutase, glutathione S-transferase, glutathione peroxidase.

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Introduction

Despite being harmful to biological systems, low levels of reactive oxygen species (ROS) have been identified as important regulators of signal transduction processes involved in cell growth and differentiation. Aerobic organisms developed a complex network of antioxidant defense system as a protection against harmful effects of ROS as well as to promote a transient action of ROS on signal transduction pathways (Valko et al., 2006; Piccoli et al., 2007). For this purpose, there are some enzymatic and non enzymatic antioxidants. Enzymatic antioxidants involve superoxide dismutase (copper zinc, CuZn-SOD and manganese containing, Mn-SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9), glutathione S-transferase (GST, EC 2.5.1.18) (Mates et al., 1999). Non-enzymatic antioxidants involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, melatonin and other compounds (MacCall & Frei, 1999).

In the context of bone metabolism, some reports have claimed that oxidative stress may play an important role in pathogenesis of postmenopausal osteoporosis (Ozgocmen et al., 2007; Sendur et al., 2009).

MC3T3-E1 cells have being employed as an excellent model for studying osteoblast differentiation triggered by different stimuli such as ascorbic acid (AA) and β-glycerol phosphate (β-GP) (Quarles et al., 1992; Franceschi et al., 1992). Since, as mentioned above, oxidative stress may be involved in bone pathogenesis and there is no information about the redox status on differentiated osteoblasts, we proposed to examine some antioxidant enzymes on MC3T3-E1 cells cultured for 7 days in the presence of AA and β-GP. In this period they differentiate into functional osteoblast-like cells, characterized by procollagen hydroxylation, processing, and fibril assembly followed by the dramatic induction of specific genes associated with the osteoblastic phenotype, including alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN) and the PTH/PTH related protein receptor (Quarles et al., 1992; Franceschi and Iyer, 1992).

In this work, we observed that at the initial stage of osteoblast differentiation, CAT, SOD and GPX presented lower activities. This finding suggests that the osteoblastic differentiation state could define the sensitivity to the oxidative damage through the reduction of the activity of antioxidant enzymes which can be relevant to bone diseases as osteoporosis.

Materials and methods

Materials and Reagents. Ascorbic acid (AA), β-glycerophosphate (β-GP), N,N-dimethylaniline (MDA), 4-aminoantipyrine (4-AAP), 1-chloro-2,4-dinitrobenzene (CDNB), 2, 6-colorimetric reagent dichloroindophenol (DCIP), N-methyl-2-phenylindole (MPO), 5, 5′-dithiobis (2-nitrobenzoate) (DTNB), were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). MC3T3-E1 preosteoblast cells were from American Type Culture Collection (ATCC, Rockville, MD). Anti-Mn-SOD was purchased from Sigma-Aldrich (St. Louis, USA), anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-rabbit and anti-goat was purchased from Cell Signaling Technology (Beverly, MA). All other chemicals and reagents used in thi study were of analytical grade.

Cell culture condition. MC3T3-E1 preosteoblast cells were routinely grown in modified alpha minimum essential medium (α-MEM) without AA, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics and incubated in a humidified atmosphere at 37 °C and at 5% CO_2. For all experiments, cells were plated at an initial density of 50,000 cells/cm^2. All treatment conditions were

performed after 24 h of cell attachment (day 0). Differentiation and mineralization were induced by adding osteogenic medium (AA/β-GP) containing 50 µg/ml AA and 10 mM β-GP. After plating, cells were treated every three or four days. Cells maintained with medium alone were used as a control (Ctrl) for all experiments.

Redox status Analysis. After 7 days in culture the cells were rinsed three times with cold, sterile, phosphate-buffered saline and harvested. Following centrifugation of the cells at 4°C and 1500 rpm for 5 min, the supernatant was discarded. The cell pellet was redissolved in 400µl of 10mM phosphate ice-cold buffer (pH 7.0) and sonicated in an ice bath for 15s. After recentrifugation at 15000 rpm for 10 min at 4°C, the supernatant were removed for assays of antioxidant enzymes, as well as malondialdehyde (MDA), GSH and protein quantification.

CAT activity was measured by H_2O_2 decomposition and monitored 30 minutes in absorbance at 230 nm of a reaction medium containing 10 mM H_2O_2, 50 mM sodium phosphate buffer (pH 7.0), and the enzyme sample (Maehly & Chance, 1957).

Total SOD activity was measured by the coupled reaction of DMA with 4-AAP under the catalysis of SOD and absorbance determined at 554 nm (Tang et al., 2002). GST activity based on the conjugation of CDNB with glutathione and the activity determined at 340 nm as described by Habig et al. (1974). GPX activity was measured at 620 nm, employing DCIP and hydrogen peroxide as the substrate by method of Hawkes & Craig (1990). Lipid peroxidation as described by Gomes-Marcondes and Tisdale (2002). MDA was determined using MPO as the substrate. The resulting absorbance was measured at 570 nm and the results were expressed in nanomoles per milligram protein. For total glutathione content was used standard curves generated with known amounts of glutathione and DTNB as substrate by Ellman (1959) assay. The absorbance was measured at 412 nm and values expressed as nmoles/µg protein. All of the antioxidant enzyme activities were expressed as ΔOD/minutes/µg protein. The analysis were measured in ELISA plate reader (Athos Labtech, Sussex, UK).

Western blotting analysis. Following treatment of cells for 7 days, the medium was aspirated and the cells were washed with cold physiological solution. Cells were then incubated in 200 µl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using Lowry method (1951). An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 75 µg of protein, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 ºC with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. The detection was made using enhanced chemiluminescence ECL. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by

volume densitometry after scanning the film (data are presented as the protein to β-actin ratio).

Statistical analysis. Results are presented as means ± standard deviation (SD). The data were analyzed by Student's *t*-test using Microcal Origin 6.0 software. Differences were considered significant when the *P* value was less than 0.05.

Results and Discussion

MC3T3 cells were cultured in presence of ascorbic acid and β-glycerolphosphate for 7 days and afterwards the activity of different enzymes and the level of GSH and MDA were examined.

Table 1 shows that CAT, SOD and GST activities were significantly lower ($P<0.05$) in differentiated cells. However, the expression of Mn-SOD determined by Western blot (Fig. 1) was 27% higher ($P<0.05$) in the differentiated compared to the undifferentiated cells. Total GSH content, which includes both reduced and oxidized glutathione, was 12% lower in the differentiated cells.

Table 1. Changes in the levels of total GSH and MDA and the activities of SOD, CAT, GST and GPX in preosteoblast cells after 7 days of treatment with AA/β-GP

Parameters	Experiments groups	
	Control	AA/β-GP
MDA	12.011±1.069	9.372±0.789
Total GSH	1.649±0.098	1.432±0.067
SOD	3.017±0.154	1.606±0.083*
CAT	0.571±0.026	0.148±0.020*
GST	6.210±0.348	5.057±0.309*
GPX	1.497±0.132	0.711±0.037*

Values are expressed as means ± SD.; n = 12 for each experimental group. (*) Mean values were significantly different, p < 0.05.
GSH, as nmoles total GSH/μg of protein
MDA = malondialdehyde as nmoles MDA/μg of protein
SOD = total superoxide dismutase (ΔOD/min/μg of protein)
CAT = catalase (ΔOD/min/μg of protein)
GST = glutathione S-transferase (ΔOD/min/μg of protein)
GPX = glutathione peroxidase (ΔOD/min/μg of protein)

Changes in the cellular detoxifying defenses against superoxide and H_2O_2 during induced differentiation have been reported, however there is no information in the literature about the redox status of differentiated osteoblasts stimulated with AA/β-GP. For instance, Chen and collaborators (2008) have reported that human mesenchymal stem cells differentiated into osteoblasts towards AA/β-GP presented an increase of mitochondria number and function. Accordingly, these authors observed an upregulation of Mn-SOD and CAT which represent and important physiological adaptation to prevent accumulation of ROS in response to the augment of mitochondrial respiratory rate.

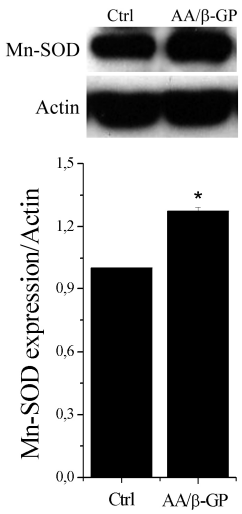

Fig1. Western blot analysis of the expression of Mn-SOD anti-oxidant enzyme in preosteoblast cells after 7 days of treatment with AA/β-GP. Detection of enzyme was realized by Western blot as described in Materials and methods. Results are representative of 3 different experiments. Values are expressed as means ± SD. (*) Mean values were significantly different, P < 0.05.

Another important observation made by different groups has been indicated that the antioxidant property of AA is not the main factor which contributes for the differentiation process. Therefore, the cellular redox status changing promoted by different levels of other antioxidants (enzymes and nonenzymatic components) seems to be more relevant for the

occurrence of osteoblast differentiation (Chen et al., 2008; Fatokun et al. 2008). Under this aspect, Varadharaj et al. (2005) examined the effects of different concentrations of ascorbic acid in lung microvascular endothelial cells. According to Varadharaj et al. (2005) studies higher concentration of AA (1 mM) resulted in reduced cell viability and morphology alteration; on the other hand, when it was employed at lower dose (0.3 mM) differentiation process was observed.

The capacity of AA to induce differentiation has been detected in other cellular types. AA-induced differentiation of leukemia cell line HL-60 (Kang et al., 2003) and AA-induced redifferentiation of human hepatoma cells (Zheng et al. 2002) were associated with an increase of SOD activity, decrease of CAT activity, increase of H_2O_2 prodution and inhibition of the proliferation.

In our study, the observation of a significant reduction in total SOD, CAT, GST and GPX activity in differentiated osteoblastic cells could explain the enhancement of sensitivity by hydrogen peroxide-induced oxidative stress during the ascorbic acid and beta-glycerophosphate-induced osteoblastic differentiation reported by Fatokun et al. (2008). Despite it, was observed a decrease of total SOD activity and increase of MnSOD expression. This changing in the MnSOD expression may be explained at least in part by alterations in mitochondrial biogenesis necessary to energy production during the initial phase of osteoblastic differentiation process, as previously reported in human mesenchymal stem cells by Chen et al. (2008).

GPX uses GSH as a cofactor to reduce H_2O_2 to H_2O molecules and organic hydroperoxide to alcohols (Mates et al., 2000; Ray et al., 2002). Dreher and collaborators (1998) demonstrated that GPX is expressed by osteoblasts and may be relevant for protection against H_2O_2 produced by osteoclasts during bone remodeling (Dreher et al., 1998). Interestingly, our findings suggest that at initial differentiation stage, this enzyme presented lower activity, with might be important for the occurrence of differentiation.

In addition, the potential oxidative stress caused by AA/β-GP-induced osteoblastic differentiation was evaluated by measuring the production of MDA, an abundant aldehyde formed from lipid peroxidation (Choi et al. 2008, Lee et al., 2008). The content of MDA remained unchanged in both experimental groups. These results suggest that, that despite the reduction of the SOD e CAT activity, the content of MDA showed not significantly change in the oxidant generation during the initial phase of osteoblastic differentiation. Importantly, we have not observed any correlation between collagen synthesis and ascorbic acid-induced lipid peroxidation, as demonstred before for in cultured human fibroblasts (Geesin et al., 1991; Houglum et al., 1991).

Our study was the first one that evaluated the antioxidant enzyme activities in the preosteoblasts cultured in presence of AA/β-GP for 7 days. In this experimental condition, it is clear that there is not induction of oxidative stress, even with the lower activity of CAT, SOD and GPX. It is an indication that the variation on the efficiency of antioxidant enzymatic antioxidant might guarantee low level of ROS, which is able to regulate signal transduction pathways, without affecting the osteoblasts functionality. Our findings also indicated that beyond the increase of the susceptibility to oxidative stress during the differentiation, the differentiated cells could also succumb to the damage of xenobiotic agents. In addition, we also can hypothesize that the osteoblastic differentiation state increases the sensitivity of these cells towards the

oxidative damage which can be relevant to management of bone disease as osteoporosis and also the development of antioxidant therapies.

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Capítulo 3

RIBOFLAVINA E SEUS FOTOPRODUTOS PROVEEM UM MICROAMBIENTE PARA A DIFERENCIAÇÃO DE OSTEOBLASTOS

“RIBOFLAVIN AND ITS PHOTOPRODUCTS PROVIDE A MICROENVIRONMENT FOR OSTEOBLASTS DIFFERENTIATION”

Riboflavin and its photoproducts provide a microenvironment for osteoblasts differentiation

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ABSTRACT Besides being a component of coenzymes, riboflavin (Rb) and its photoproducts appear as modulators of signal transduction cascades. Also this vitamin uptake has been proved to ameliorate the osteoporosis. Therefore, the main goal of this study was to investigate the effect of Rb and irradiated riboflavin (IRb) *in vitro*, using as an experimental model, preosteoblast cells which are considered a good model to evaluate the osteogenesis process. For this purpose, MC3T3-E1 preosteoblasts were exposed to subtoxic concentration of Rb and IRb (5 μM) for different periods and mediators of signaling pathways related to survival, apoptosis, differentiation, osteoclastogenesis and osteoblastogenesis processes were examined by western blotting or PCR. Rb and IRb caused cell cycle arrest at G0/G1 phase and accordingly inhibited AKT kinase, a proliferation mediator. Flavins caused differentiation of preosteoblast cells as evidenced by increase of osteocalcin, osteopontin and BMP2. In addition, higher MMP-9 and -2 activities were observed. Importantly, the capacity of flavins to trigger osteoblasts differentiation was also reinforced by upregulation of connexin 43, down regulation of caveolin-1 and negative modulation of Notch cascade. This study brought out strong evidences that high concentration of Rb and IRb generates an osteogenic microenvironment through modulating different mediators of signaling pathways. In summary, this study pointed out the potential application of Rb and its photoproducts in osteoblasts phenotype development and consequently an alternative therapeutic adjuvant of osteoporosis.

Keywords: Riboflavin; Osteoblast; Antioxidant enzymes; Caspase; Metalloproteinases; Osteocalcin

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1. Introduction

Riboflavin (Rb) is the precursor of essential flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes participate in numerous enzymatic processes dependent on electron transfer reactions that occur in energy-producing, biosynthetic, and detoxifying and electron-scavenging pathways [1]. Besides being important component of coenzymes, other biological properties of riboflavin have been described. For instance, we provided evidence that photoderivatives of riboflavin have strong activities in haematological malignancy [2] and human prostate cancer cells [3]. Increase dietary riboflavin and pyridoxine intake has been associated with higher bone mineral density in elderly men and women [4]. Pyridoxine and certain other B vitamins (riboflavin, folate and cobalamin) supplementation during the senility has directed attention, since it was observed a maintenance of low homocysteine levels [4, 5, 6], which is considered a novel risk factor for age-related osteoporotic fractures, mainly in women homozygous for the methylentetrahydrofolate reductase (MTHFR) 677 T allele [5]. Researches indicate that whole bone status improvement with prolonged antioxidant vitamin supplementation, might be used as a palliative treatment for osteoporosis [7, 8]. However, the direct effect of Rb and its photoderivatives on osteoblast has not been examined.

In this paper the effects of Rb and irradiated riboflavin (IRb) were investigated in the MC3T3-E1 pre-osteoblastic cell line, a well-accepted model of osteogenesis *in vitro* characterized for the induction of specific genes associated with the osteoblastic phenotype, including type I collagen, alkaline phosphatase, osteopontin and osteocalcin during the differentiation process [9, 10].

Our results showed that high concentration of Rb and IRb generates an osteogenic microenvironment through changes of redox status and modulation of signaling pathways. Furthermore, in the absence of osteogenic inductors, Rb and IRb negatively regulated the expression of protein associated with the suppression of osteoblast differentiation. This study provided strong evidences about the potential application of Rb and its photoproducts in osteoblasts phenotype development and consequently its use as an alternative therapeutic adjuvant in osteoporosis treatment.

2. Methods and Materials

2.1. Materials and Reagents

Ascorbic acid (AA), β-glycerophosphate (β-GP), riboflavin (Rb), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N,N-dimethylaniline (MDA), 4-aminoantipyrine (4-AAP), 1-chloro-2,4-dinitrobenzene (CDNB), 2, 6-colorimetric reagent dichloroindophenol (DCIP), N-methyl-2-phenylindole (MPO), 5, 5′-dithiobis (2-nitrobenzoate) (DTNB), p-nitrophenylphosphate, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red), were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). MC3T3-E1 preosteoblast cells were from American Type Culture Collection (ATCC, Rockville, MD). Polyclonal antibodies against Connexin 43, Caveolin-1, phospho-ATF-2 Thr71, phospho-AKT Thr308, phospho-p70 S6 kinase Thr389, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-PP2A Tyr307, Bcl2, Bax, OPG, RANKL and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP-1 (p116/p25), β-catenin were from Upstate Biotechnology, Inc. (Lake Placid, NY). Caspases 3, 8 and 9 Colorimetric Assay Kits were obtained from R&D Systems (Minneapolis, MN). All other chemicals and reagents used in this study were of analytical grade.

2.2. Cell culture condition

MC3T3-E1 preosteoblast cells were routinely grown in modified alpha minimum essential medium (α-MEM) without AA, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics and incubated in a humidified atmosphere at 37 °C and at 5% CO_2. For all experiments, cells were plated at an initial density of 50,000 cells/cm^2. All treatment conditions were performed after 24 h of cell attachment (day 0). After plating, cells were treated every three or four days. Cells maintained in medium supplemented with FBS plus antibiotics were used as a control (Ctrl) for all experiments.

2.3. Riboflavin irradiation

Solution of 250 μM Rb in α-MEM medium at the pH 7.44 (15 mL) was placed in a Petri dish and irradiated with UVC light ($\zeta_{máx}$=253.5 nm) for 30 min; the lamp was placed 40 cm from the Rb solution. Alpha-MEM medium was irradiated in the same conditions and used as a control. After irradiation the percentage of Rb and its photoproducts was the following, as determined by mass spectrometry: 79% of riboflavin, 6.2% of lumichrome, and 14.8% composed of formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin [3].

2.4. Treatment of cells with irradiated riboflavin

Cells were plated at an initial density of 50,000 cells/cm^2 in 96-well tissue-culture plates. All treatment conditions were performed after 24 h of cell attachment (day 0) and then treated with different concentrations of riboflavin (Rb) or irradiated riboflavin (IRb) (5–50 μM final concentrations) for 24 h. Cell viability was assessed by the MTT reduction, nucleic acid content and neutral red uptake assays.

2.5. Nucleic Acid Content

Cell number in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. [11]. Cells were washed twice with cold (PBS) and a soluble nucleotide pool was extracted with cold ethanol. Cell layers were then dissolved in 0.5 M NaOH at 37°C/1 h. Absorbance at 260 nm of the NaOH fraction was used as an indicative of cell number. Results are expressed as mean percentage of absorbance at 260 nm in treated wells compared with controls.

2.6. MTT reduction assay

Medium containing irradiated riboflavin was removed and 0.2 ml of MTT solution (0.5 mg MTT/ml of culture medium) was added to each well. After incubation for 4 h at 37 ºC, the medium was removed and the formazan released by solubilisation in 0.2 ml of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance measured at 570 nm [12].

2.7. Neutral Red Uptake

NRU assay was performed according to the method of Borenfreund and Puerner [13]. After 4 h of incubation with serum-free medium containing 50 μg neutral red/ml, the cells were washed quickly with PBS and then 0.1 ml of a solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After shaking on a microtitre plate shaker the absorbance was read at 540 nm.

2.8. Determination of Cell Cycle and Apoptotic Cell Death with Fluorescence-activated Cell Sorter (FACS)

MC3T3-E1 cells were plated on 6 cm diameter dishes at a density of 50,000 cells/cm^2 and cultured in the presence of 10% FBS medium for 24 h. For serum deprivation experiments cells were washed three times in PBS and cultured in serum free αMEM. After 24 h of serum starvation, cells were treated with 5 μM of Rb or IRb for 24 h. After this period the cells were harvested and deattached by trypsinization. Cells were washed with cold PBS and resuspended in paraformaldehyde 2% in PBS at 4 °C. Subsequently, 1 ml of propidium iodide solution was added and maintained at 4ºC for 15 min. The analysis was

performed in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA,USA). It was analyzed 10,000 events per sample. DNA content was evaluated using a FL2 detector in a linear scale. To eliminate cell aggregates, the cell population to be analyzed was selected from a bivariate histogram showing the area (FL2A) *versus* the width (FL2W) of the signal FL2. The analysis of cell percentage in the different phases of the cell cycle (Sub-G_1, G_0/G_1, S/M) was performed using the CellQuest 3.4 software (BD Biosciences, San Jose, CA, USA).

2.9. Cell proliferation assay

Cell number was determined by haemocytometer counts after trypsinization and represented as the means ± standard deviation (SD) of triplicates.

2.10. Alkaline phosphatase activity assay

Alkaline phosphatase activity was measured in the total cellular lysate after sonication in buffer containing 1 mM Tris (pH 8.8), 0.5% Triton X-100, 10 mM Mg^{2+} and 5 mM p-nitrophenylphosphate as a substrate. The reaction was stopped with 1M NaOH and the absorbance measured at 405 nm.

2.11. Collagen staining and semi-quantitative analysis

Pre-osteoblast cell differentiation into matrix-producing osteoblasts was assessed by quantification of collagen production using Picrosirius Red staining as previously described (Tullberg-Reinert and Jundt, 1999).

2.12. mRNA expression of matrix proteins and osteoblast markers

Total RNA was isolated from cells using Trizol reagent (Sigma-Aldrich Zwijndrecht, Netherlands) according to manufacturer's instructions. Reverse transcription was performed on 5 ng of total RNA using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 µl. Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Invitrogen, Breda, Netherlands) on the Biometra PCR system. PCR primers for mice were selected from multiple exons: osteopontin (NM_009263.1), 5′-tctgatgagaccgtcactgc-3′ and 3′-cacccgagagtgtggaaagt-5′; osteocalcin (NM_007541.2), 5′-gcgctctgtctctctgacct-3′ and 3′-gccggagtctgttcactacc-5′; β-actin (NM_007393), 5′-tctcttccagccctctttca-3′ and 3′-atggtggtgcctccagatag-5′. The cycling program was 94°C for 2 min, 58°C for 60 seconds, 72°C for 60 seconds for the first cycle and 94°C for 30 sec, 58°C for 60 seconds, 72°C for 60 seconds for 30 cycles. PCR products were analyzed by 3% agarose gel electrophoresis and stained with ethidium bromide. The level of mRNA expression is expressed relative to the β-actin level. All experiments were performed in triplicate.

2.13. Redox status Analysis

After a further 7 days, to prepare cell cultures for assays, the cells were rinsed three times with cold, sterile, phosphate-buffered saline and harvested. Following centrifugation of the cells at 4°C and 1,500 rpm for 5 min, the supernatant was discarded. Cell pellet was resuspended in 400µl of ice-cold 10mM phosphate buffer (pH 7.0) and sonicated in an ice bath for 15s. After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatant was removed for assays of antioxidant enzymes, as well as malondialdehyde (MDA), total GSH and protein quantification. CAT activity was measured by H_2O_2 decomposition and monitored 30 minutes in absorbance at 230 nm of a reaction medium containing 10 mM H_2O_2, 50 mM sodium phosphate buffer (pH 7.0), and the enzyme sample [14]. Total SOD activity was measured by the coupled reaction of DMA with 4-AAP and absorbance determined at 554 nm [15]. GST activity based on the conjugation of CDNB with glutathione and the activity determined at 340 nm as described by Habig et al. [16].

GPX activity was measured at 620 nm, employing DCIP and hydrogen peroxide as the substrate by method of Hawkes & Craig [17]. Lipid peroxidation as described by Gomes-Marcondes and Tisdale [18]. MDA was determined using MPO as the substrate. The resulting absorbance was measured at 570 nm and the results were expressed in nanomoles per milligram protein. For total glutathione content was used standard curves generated with known amounts of glutathione and DTNB as substrate [19]. Absorbance was measured at 412 nm and values expressed as nmoles/µg protein. All of the antioxidant enzyme activities were expressed as ΔOD/minutes/µg protein. The absorbance was measured an ELISA plate reader (Athos Labtech, Sussex, UK).

2.14. Western blotting analysis

Following treatment of cells for 7 days, the medium was aspirated and the cells were washed with cold physiological solution. Cells were then incubated in 200 µl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using Lowry method [20]. An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 75 µg of protein, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 ºC with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. Bands detection was made using enhanced chemiluminescence ECL. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to β-actin ratio).

2.15. Caspases 3, 8 and 9 activity assays

Caspases activities were determined by the measurement at 405 nm of p nitroaniline (pNA) released from the cleavage of Ac-DEVD-pNA, IETD-pNA and LEHD-pNA as substrates of caspases 3, 8 and 9, respectively. Enzyme activities were expressed in pmol/min and the extinction coefficient of pNA was 10,000 $M^{-1}cm^{-1}$.

2.16. Zymographic analysis

Proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by de Souza et al. [21]. After the treatment the culture medium was collected and stored at -20 ºC in the presence of 1 mM PMSF (phenyl-methylsulphonyl fluoride-serine-protease enzyme inhibitor). Samples were diluted in non-reducing buffer (0.1 M Tris–HCl, pH 6.8, 20% glycerol, 1% SDS and 0.001% bromophenol blue). Volume of the samples loaded was proportional of the sample protein and resolved by SDS–polyacrylamide gel (10%) and 4% gelatin. Protein renaturation was performed in 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris–HCl and 10 mM CaCl2 (pH 7.4) at 37 ºC for 18 h. Gels were stained with 0.5% Coomassie blue G 250 for 30 min and then washed in a 30% methanol and 10% glacial acetic acid solution. Image analysis was performed on a

PC computer using ScionImage software, freely available on the internet from Scion Corporation at http://www.scioncorp.com.

2.17. Statistical analysis

Results are expressed as mean ± S.D. with at least three replicates in each group. Differences were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *P* values < 0.05 were considered significant. All data were analyzed using GraphPad Prism Software, Version 4.0.

3. Results

3.1. Riboflavin and Irradiated Riboflavin do not affect preosteoblast cells viability

To determine the possible toxic effects of IRb in preosteoblast cells, the cells were treated with Rb and IRb concentrations up to 50 µM and the effect on cell viability was determined employing the MTT reduction, neutral red uptake and nucleic acid content assays. Cell viability was not affected neither for Rb or IRb treatments (Fig. 1A e 1B).

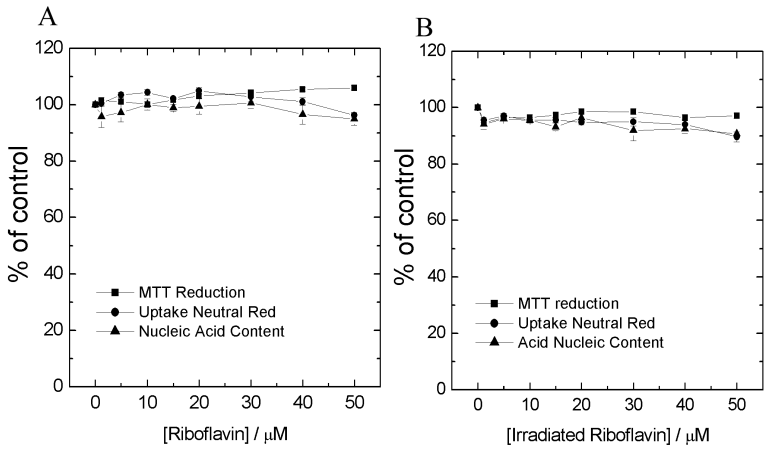

Fig. 1. Riboflavin and Irradiated Riboflavin do not affect preosteoblast cells viability. Cells were treated with different concentrations of riboflavin (A) and irradiated riboflavin (B). Cell viability was evaluated after 24 h. In the absence of Rb and IRb, the MTT reduction, incorporation of neutral red and acid nucleic content were considered as 100%. Each experiment was performed in a 96 wells plate and results represent the means ± D.S. of 3 experiments run in triplicate.

3.2. Riboflavin and Irradiated Riboflavin intensified G0/G1 arrest in preosteoblast cells

To determine whether Rb or IRb might affect the cell cycle progression, we conducted FACS cell-cycle analysis. Although hypodiploid DNA peaks are commonly found in the case of apoptosis, Rb and IRb did not produce a significant increase in the proportion of hypodiploid cells up to 24 h (Table 1). We found that Rb and IRb caused G_0/G_1 arrest in MC3T3-E1 cells as early as 24 h after the start of treatment (Table 1).

Table 1. Evaluation of cell cycle progression in preosteoblastic MC3T3-E1 after treatment with riboflavin and irradiated riboflavin

Cells	Sub-G_1	G_0/G_1 phase	Synthesis phase/Mitosis
Control	1.36 ± 0,30	39.60 ± 1.46	59.05 ± 1.17
Rb	1.77 ± 0.95	45.52 ± 1.71*	52.70 ± 0.95*
IRb	1.34 ± 0.24	47.57 ± 1.57*	51.09 ± 1.34*

After 24 h of serum starvation cells were treated with 5 µM riboflavin or 5 µM irradiated riboflavin for 24 hours. Cells were stained with propidium iodide and the DNA content was analyzed by flow cytometry. Values of the percentages of MC3T3-E1 remaining in the Sub-G1, G0/G1 phase and Synthesis/Mitosis phase are shown on table. Results represent the means ± SEM of three independet experiments. * P<0.05 vs. Control and # P<0.05 vs. Riboflavin

3.3. Effects of Riboflavin and Irradiated Riboflavin on temporal sequence of preosteoblast development

Rb and IRb did not significantly affect MC3T3 cells growth (Fig. 2A). Importantly, cells cultured in the

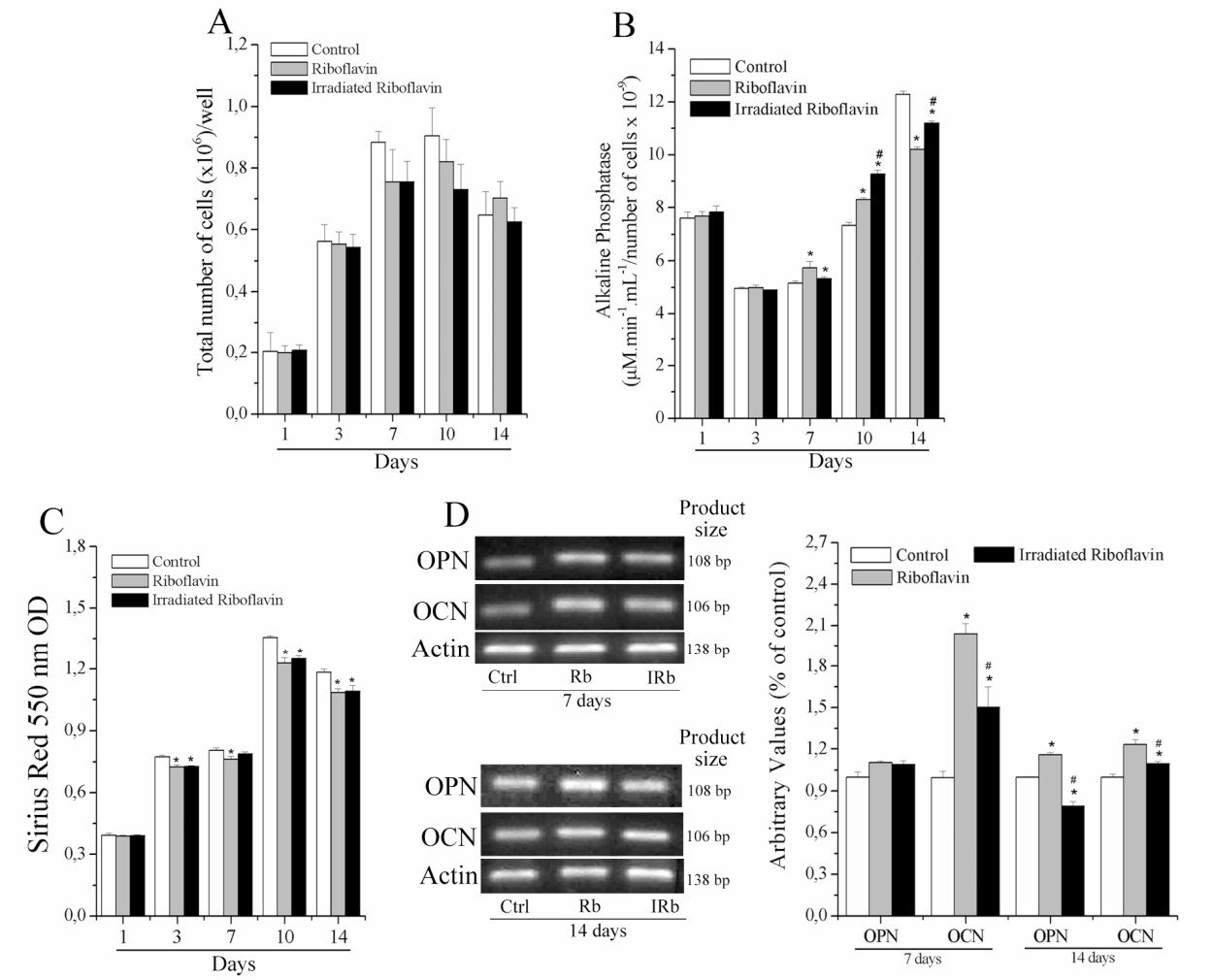

Fig. 2. Time course response of MC3T3-E1 cells to Rb and IRb. (A) Cell number was assessed by direct counting after the stated time of culture; (B) Alkaline phosphatase activity during the treatment was measured using p-nitrophenylphosphate as a substrate; (C) Type I collagen production by the culture as assessed by Picrosirius staining; (D) mRNA expression of OCN and OPN in the cultures at the 7 days and 14 days as analyzed by RT-PCR. MC3T3-E1 cells were treated with 5 μM of riboflavin or irradiated riboflavin. All treatment conditions were performed after 24 h of cell attachment (day 0). Measurements were taken during 14 days of treatment. Each data value represents the mean result of triplicate or quadruplicate samples from a single representative trial; error bars represent the standard deviation of these samples. Experiments were repeated at least twice. * P<0.05 vs. Control and # P<0.05 vs. Riboflavin.

presence of Rb and IRb, displayed a profile of alkaline phosphatase activity very similar to the control (Fig. 2B). The effect of Rb and IRb on collagen content in preosteoblastic cells is shown in Fig. 2C. Rb or IRb 5 μM seems to attenuate the increase in collagen content in all period ($P < 0.05$).

After 7 days of treatment, Rb and IRb increases mRNA expression of OCN, a master marker of late stage of the osteoblastic differentiation ($P < 0.05$) and in the same period OPN mRNA expression, a master marker of early stage of the osteoblastic differentiation, showed a discrete increase in the Rb and IRb groups (Fig. 2D). After 14 days in culture, the preosteoblast maintained high OCN mRNA levels, however less expressive than observed at day 7 ($P < 0.05$). Rb maintained the increase of OPN mRNA at 14 days of cultures ($P < 0.05$), while the IRb promoted the reduction of OPN mRNA compared to control and Rb ($P < 0.05$) (Fig. 2D).

3.4. Irradiated Riboflavin alters activities of some scavenging system enzymes and total GSH levels

To investigate the alterations of scavenging system enzymes and biomarkers for oxidative stress status in preosteoblast cells after 7 days of Rb and IRb treatment, we analyzed SOD, CAT, GST and GPX activities, total GSH content and the levels of MDA which is a marker of lipid peroxidation (Table 2). No significant change in total SOD activity and MDA level were observed. IRb raises the level of total intracellular GSH, which controls the cellular redox state as a substrate of GPX and GST. GST activity was significantly higher (40%, $P < 0.05$) in the IRb treatment than in the control. After 7 days of treatment a marked reduction of CAT (57%) and GPX (58%) activities was observed in the IRb compared to the control. The relatively stable level of MDA showed that Rb and IRb treatment does not cause oxidative stress despite the changes of antioxidant enzymes. The significant increase of total GSH content and GST activity suggest a possible increase in the cellular antioxidative capacity resulting from the IRb treatment.

Table 2. Changes in the levels of total GSH and MDA and the activities of SOD, CAT, GST and GPX in preosteoblast cells after 7 days of treatment with 5 µM of riboflavin or irradiated riboflavin

Parameters	**Experiments groups**		
	Control	**Rb**	**IRb**
MDA	12.011±1.069	13.348±1.080	11.630±0.892
Total GSH	1.649±0.098	1.981±0.126	2.148±0.178*
SOD	3.017±0.154	2.756±0.296	2.843±0.199
CAT	0.571±0.026	0.287±0.061*	0.248±0.046*
GST	6.210±0.348	6.592±0.363	8.716±0.348*#
GPX	1.497±0.132	1.262±0.102	0.628±0.066*#

Values are expressed as means ± SD.; n = 12 for each experimental group. (*) Mean values were significantly different, $p < 0.05$.
GSH, as nmole GSH/µg of protein
MDA, as nmole MDA/µg of protein
SOD = superoxide dismutase (ΔOD/min/µg of protein)
CAT = catalase (ΔOD/min/µg of protein)
GST = glutathione S-transferase (ΔOD/min/µg of protein)
GPX = glutathione peroxidase (ΔOD/min/µg of protein)

3.5. Effect of Rb and IRb on the expression of bone anabolic factor, osteoclast-inducing factor and proteins associated with bone homeostasis

To identify potential alterations in the modulation of bone metabolism after Rb and IRb treatment, we investigate the expression of bone anabolic factor (BMP2), osteoclast-inducing factor (OPG and RANKL) and proteins associated with bone homeostasis (Connexin 43 and Caveolin-1) in MC3T3 cells. Significant increase of Connexin 43, BMP2 and RANKL was observed after 7 days of the IRb treatment ($P < 0.05$), while Rb increased the expression of Connexin 43 and BMP2 (Fig. 3). Compared to the control, Rb and IRb treatment also showed a significant reduction expression of Caveolin-1 and OPG ($P < 0.05$) (Fig. 3).

3.6. Rb and IRb suppress survival pathways and activated caspases 8 and 3

The previously described data suggest the effect of the irradiated riboflavin in the activity of antioxidant enzymes. Changes in the redox state of cells are thought to induce modifications of cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors. Given the general importance of kinases and phosphatases in the regulation of cellular survival, proliferation and differentiation of osteoblasts, we decided to investigate the serine-threonine protein kinase Akt, p70 S6 K and serine/threonine phosphatase PP2A activities after treatment with Rb and IRb. MC3T3-E1 cells were treated with 5 µM of Rb and IRb for 7 days, which caused inhibition of AKT/p 70 S6 K, and activation of PP2A by dephophorylating the inhibitory Y307-residue (Fig.4A).

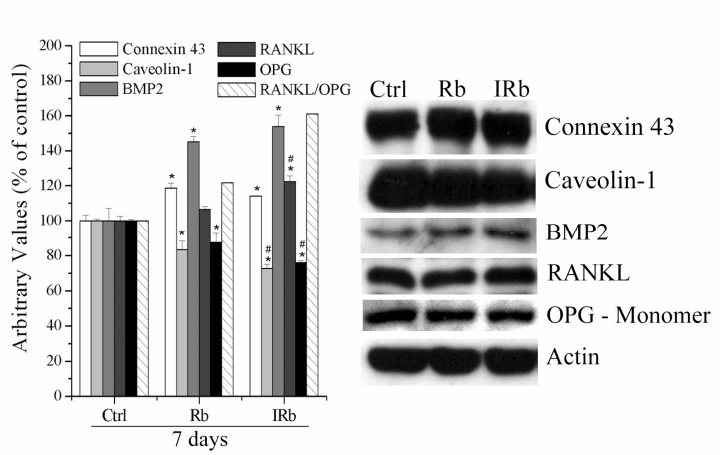

Fig. 3. Effect of Rb (5 µM) and IRb (5 µM) on expression of bone anabolic factor (BMP2), osteoclast-inducing factor (RANKL/OPG) and proteins associated with bone homeostasis (Connexin 43 and Caveolin-1) after 7 days of treatment. Detection of proteins was performed by Western blot as described in materials and methods. Results are representative of 3 different experiments. Values are expressed as means ± SD. * *P*<0.05 *vs.* Control and # *P*<0.05 *vs.* Riboflavin.

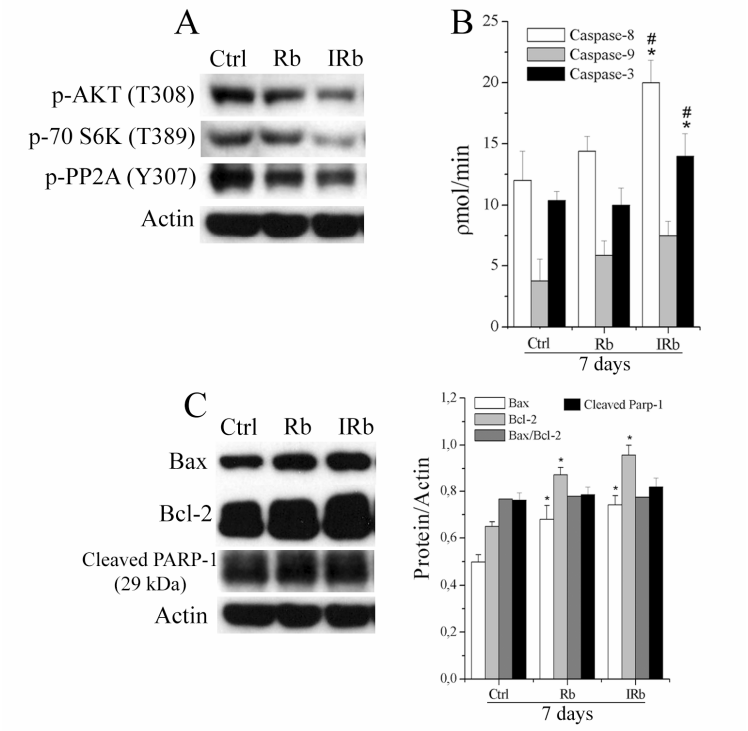

Fig. 4. Rb and IRb suppress survival pathways and induce changes in the caspases activity. MC3T3-E1 cells were treated at day 7 with 5 µM of Rb or IRb. (A) Analysis of cellular survival proteins by targeted AKT/p70 S6 K and activity of PP2A, a negative regulator of survival pathway. (B) Western blotting analysis of Bcl-2, Bax and cleaved PARP-1 levels and quantitative analysis of Bax/Bcl-2. Quantification of immunoband intensities from Western blotting analysis was determined by densitometric scannig. Data were expressed as Bax/Bcl-2 ratio. (C) Colorimetric assay was performed to determine the activation of caspase-8, caspase-9 and caspase-3. Detection of proteins was performed by Western blot as described in materials and methods. Results are representative of 3 different experiments. Values are expressed as means ± SD. * *P*<0.05 *vs.* Control and # *P*<0.05 *vs.* Riboflavin.

Survival signals are required to suppress the default pathway of apoptosis. The ultimate outcome of apoptotic signaling is the release of factors from the mitochondria into the cytosol. To investigate whether the survival pathway (AKT/p70 S6 K) suppression by Rb and IRb treatment promoted the activation of apoptosis pathway, we examined caspases (caspase-8, caspase-9 and caspase-3) that are crucial initiators or effectors in the cell death and differentiation pathways in osteoblasts. The participation of mitochondrial pathway of apoptosis induction was investigated by Bax/Bcl-2 ratio. After 7 days of treatment, IRb induced potent activation of caspase-8 and caspase-3 compared with the control and Rb groups ($P < 0.05$) (Fig. 4B). However, the caspase-9 activation was not expressive in both groups (Fig. 4B). In addition, Rb and IRb did not alter the Bax/Bcl-2 ratio (Fig. 4C). Activation of caspases during apoptosis results in the cleavage of critical cellular substrates. Although we observed difference on caspase-8 and caspase-3 activities in the Rb group, no difference was observed in the cleaved PARP-1, a well established caspases substrate (Fig. 4C).

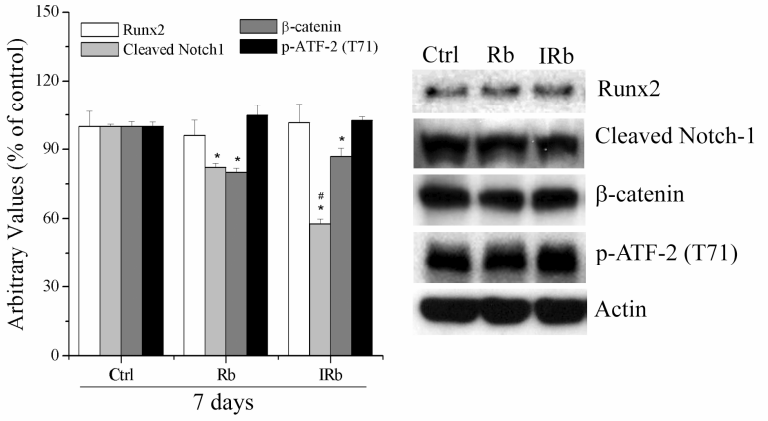

Fig. 5. Rb (5 μM) and IRb (5 μM) attenuate the cleavage of membrane-bound transcription factor (Notch-1) that impairs osteoblastic differentiation. Detection of proteins was performed by Western blot as described in materials and methods. Results are representative of 3 different experiments. Values are expressed as means ± SD. * $P<0.05$ *vs.* Control and # $P<0.05$ *vs.* Riboflavin.

3.7. Rb and IRb decrease the cleavage/activation of Notch-1 and the active β-catenin levels

Differentiation of MC3T3-E1 preosteoblasts is associated with temporal changes in the expression of specific transcription factors. The increase of osteoblast-specific transcription factors and the reduction of the suppressor transcriptional factors are required during the development of osteoblastic phenotype and are responsible for the modulation of differentiation osteoblast markers gene expression. We then evaluated whether Rb and IRb affected osteoblast phenotype by expression and activation of transcription factors after 7 days of culture. Rb or IRb did not cause activation of ATF-2 (Fig. 5). In addition, Runx2 expression level remained unchanged (Fig. 5).

Notch signaling pathway has been associated with the inhibition and/or suppression of osteoblast differentiation. We next sought to determine whether Nocth-1 cleavage/activation was altered after Rb and IRb treatment. We observed a significant reduction of Notch-1 cleavage/activation after both treatment, however the alteration was more accentuated in the IRb group ($P < 0.05$) (Fig. 5) In the canonical Wnt/β-catenin pathway, activation of receptors results in the

stabilization of β-catenin and its subsequent translocation into the nucleus where it drives the transcription of target genes related to the osteoblastic phenotype. In order to establish if the Wnt/β-catenin pathway was affected by riboflavin, we analyzed the level of active β-catenin (dephosphorylated). After 7 days of treatment was observed a significant decrease of β-catenin level in cells treated with Rb and IRb ($P < 0.05$) (Fig. 5).

3.8. MMP2 and MMP9 activities are modulated by Rb and IRb treatment

Matrix metalloproteinases are key mediators in extracellular matrix remodelling and primarily implicated in bone growth. Increased MMP expression can occur during injury, inflammation, cellular transformation, and oxidative stress. Effect of Rb and IRb in metalloproteinases 2 and 9 activities was analyzed after 7 and 14 days of MC3T3 cells treatment. MMP9 activity showed an increase in both treatment periods ($P < 0.05$) (Fig. 6), however it was more expressive in the IRb group. A slight increase in MMP2 activity was observed in 7 and 14 days only in response to IRb treatment ($P < 0.05$) (Fig. 6).

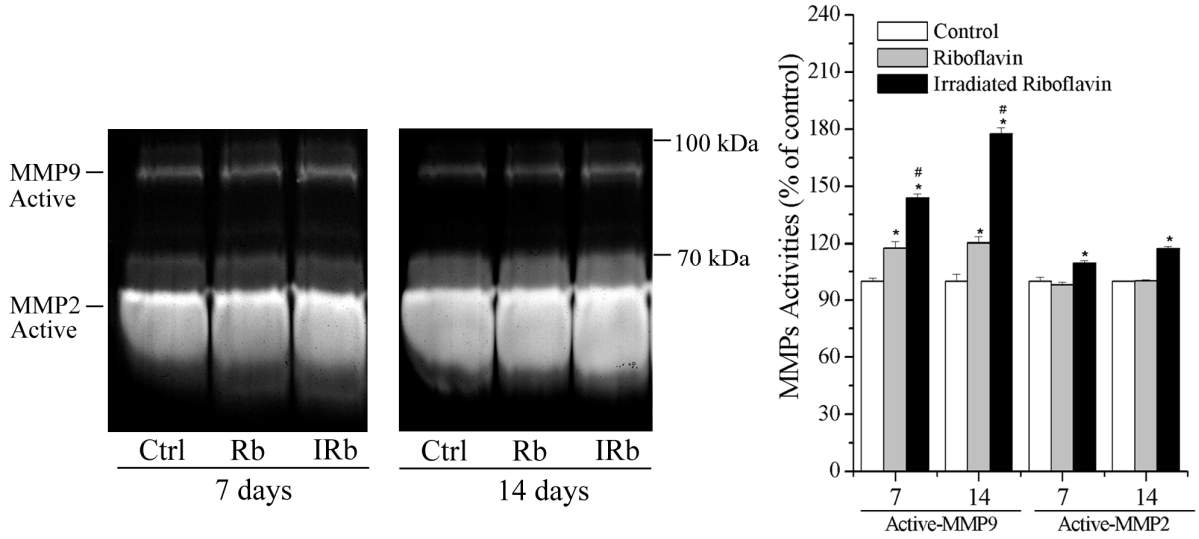

Fig. 6. Effect of Rb and IRb on MMP-2 and MMP-9 activities. Representative zymography of conditioned media from MC3T3-E1 treated with 5 μM of Rb and IRb for 7 and 14 days. The loading medium volume on the gel was normalized by the protein samples. Shown is the mean of three independent experiments ± S.D. * $P<0.05$ *vs.* Control and # $P<0.05$ *vs.* Riboflavin.

4. Discussion

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass which results in a markedly increased risk for atraumatic fractures [22]. This pathology is influenced by genetic and environmental factors such as diet and lifestyle. Therefore it is important to develop strategies and recommendations not only for the treatment but also for the prevention of osteoporosis. Agents for the treatment of osteoporosis are classified as either antiresorptive or anabolic. Antiresorptive agents work by inhibiting the activity of osteoclast and, therefore, reducing bone resorption, while anabolic agents act by stimulating formation of new bone [23]. Scientist became highly interested in nutrients, which have a potential to prevent or minimize the risk to develop osteoporosis [24, 25, 26]. Recent evidence has shown that ROS may be involved in the pathogenesis of bone loss-related diseases. Reduced bone formation is associated with

increased oxidative stress in aged men and women [7]. Marked decrease in plasma antioxidants is found in aged osteoporotic women [8]. Researches indicate that the whole bone status improved with prolonged antioxidant vitamin supplementation, which can be used as a palliative treatment for osteoporosis [7, 8]. A challenge in the research is to identify compounds that contribute to the maintenance of bone health and further characterize its mechanism of action.

Low dietary riboflavin predicts increased fracture risk in postmenopausal women homozygous for the methylentetrahydrofolate reductase (MTHFR) 677 T allele [5], while the increase of riboflavin and pyridoxine dietary intake was associated with higher femoral neck bone mineral density [4]. Pyridoxine and certain other B vitamins (riboflavin, folate and cobalamin) supplementation during the senility has directed attention, since it was observed a maintenance of low Hcy levels [4, 5, 6], which is considered a novel risk factor for age-related osteoporotic fractures, mainly in women homozygous for the methylentetrahydrofolate reductase (MTHFR) 677 T allele [5].

Despite the considerations mentioned above, the molecular effect of high riboflavin concentrations in osteoblast cells has not been described and discussed. Therefore, in this study the biological effects of riboflavin and its photoproducts (formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin), were evaluated using preosteoblast cells. MC3T3-E1 preosteoblast cell line is considered an excellent cell differentiation model that simulates the events of early osteoblastogenesis [10].

Growth and differentiation factors contained in the culture medium stimulate preosteoblast cells to undergo a developmental sequence that includes proliferation of undifferentiated precursors of osteoblasts, which subsequently differentiate into post-mitotic osteoblasts capable of expressing the osteogenic phenotype. The finding showed that Rb and IRb attenuated the anabolic metabolism of preosteoblasts cells by decreasing of the type I collagen synthesis. However, Rb and IRb, at concentration of 5 μM, increased the mRNA level of OCN. These findings prompted us to investigate the influence of Rb and IRb in different mediators of signaling pathways related to survival, apoptosis, differentiation, osteoclastogenesis and osteoblastogenesis processes.

Preosteoblast cells treated with Rb or IRb presented inhibition of AKT and P70 S6 kinase. Serine/threonine kinase AKT is an important regulator of cell proliferation and death/survival pathways. Phosphorylation of AKT at regulatory residues Thr-308 and Ser-473 leads to its full activation. The protein phosphatase 2A (PP2A) has long been known to negatively regulate AKT activity [27]. In agreement, we found that unlike observed in tumor cells [2, 3], preosteoblasts displayed activation of PP2A after both Rb and IRb treatments. Consistent with our findings, the inhibition of survival pathway have been seen related with differentiation process of B16 melanoma [28], human promyelocytic HL60 leukemia cell line [29], myogenic cells line (C2C12) [30] and accelerates chondrocyte terminal differentiation [31]. On the other hand, PI3K/AKT has been required during BMP2-induced osteoblastic differentiation [32].

Another point explored in this study was the expression/activity of potential apoptotic effectors in cell differentiation. Activation of caspases is required for BMP-4 osteoblastic differentiation MC3T3-E1 [33], while Miura et al. [34] demonstrated that caspase-3 is crucial for the differentiation of bone marrow stromal stem cells by influencing TGF-beta/Smad2 pathway and cell cycle progression. Our results showed strong activation of caspases 3 and 8 when compared to caspase 9, indicating that the intrinsic pathway is not

the major signaling pathway responsible for Rb and IRb effects in the preosteoblasts. In agreement with this notion, significant changes were not observed in the Bax/Bcl-2 ratio. Thus, these results demonstrate specific caspase activation by the extrinsic pathway in preosteoblast cells in response to irradiated riboflavin. Similar findings were obtained with HL60 cells [2].

Each cell is characterized by a particular redox status in different cellular compartments. Cellular redox status and its oscillation might define the cellular fate, such as differentiation [35, 36]. Changes in the redox status of cells are thought to be responsible for modulating cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors. Therefore, in this study we evaluated whether the effects of Rb and IRb supplementation could be related to alteration in preosteoblasts redox status. Rb and IRb at 5 μM concentration failed to alter MDA level and SOD activity after 7 days of treatment. In contrast, total CAT and GPX activities dropped in the presence of IRb. However, it was observed an augment of the total GSH level and GST activity, indicating an adaptive mechanism to the treatment. Production of GSH is considered one of the first line defenses against oxidative damage and free radical generation where GSH functions as a scavenger and cofactor in metabolic detoxification of reactive oxygen species [37]. GSH is the major cytosolic low molecular weight sulfhydryl compound that acts as a cellular reducing reagent and a protective reagent against numerous toxic substances including most inorganic pollutants, through the -SH group [38]. GSH levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis; however, a severe oxidative stress may suppress GSH levels due to the loss of adaptive mechanisms and the oxidation of GSH to GSSG. Indeed, we have noted by measuring MDA, an apparent lack of oxidative stress induction in preosteoblast cells after the treatment, indicating that probably Rb and IRb induced the biosynthesis of GSH to prevent an oxidative stress. In our investigation GPX and CAT activities were affected following preosteoblasts exposure to IRb. GPX uses GSH as a cofactor to reduce H_2O_2 to H_2O molecules and organic hydroperoxide to alcohols [39, 40], while GST catalyses the conjugation of GSH with peroxidised lipids and xenobiotic substrates in the detoxification cellular. As we have observed differentiated osteoblasts characteristic acquisition after treatment with flavins, the reduction of CAT and GPX may be associated with the differentiation cellular events. According to our data, the down-regulation of antioxidant enzymes has been described during the differentiation process of muscular cells [41] and leukemia cell line HL-60 [42] and consequently increase of susceptibility of oxidative injuries. Our data also pointed out the existence of an adaptive mechanism associated with an increase of GST activity, following the treatment of IRb. This can be due to the photoproducts generated are markedly more hydrophobic as compared to the parent compound riboflavin, therefore the results suggest that in high concentration into cell the photoproducts might be metabolized as xenobiotic substrate by GST.

It is known that bone volume is maintained by two phase of bone remodeling, namely bone formation by osteoblasts and bone resorption by osteoclasts [43]. Decrease in bone mass is due to an increased bone resorption and reduced bone formation. Bone resorption and formation are tightly coupled; therefore the inhibition of resorption eventually results in inhibition of formation. Agents for the treatment of osteoporosis are classified as either antiresorptive or anabolic. Antiresorptive agents work by inhibiting the activity of osteoclasts and, therefore, reducing bone resorption, while anabolic agents have the capacity to increase bone mass to a greater degree than antiresorptive

agents. The recent interest in bone quality has arisen from observations that the traditional measure of bone strength in clinical practice, namely bone densitometry, does not always reliably predict fracture risk [44]. Bone quality describes aspects of bone turnover, microarchitecture, mineralisation, microdamage and the composition of bone matrix and mineral that contributes to bone strength independently of bone mineral density. These aspects have been taking in consideration for developing new anabolic drugs. In this context, flavins may have a new function in the osteoporosis therapy providing a suitable microenvironment during osteoblast differentiation. Despite reduction of anabolic metabolism of preosteoblasts cells by decreasing of the type I collagen synthesis, a dramatically augment in osteocalcin and osteopontin, markers of early and late stages of differentiation during bone formation, respectively [45, 46], was detected after 5 µM Rb and IRb treatment. These findings suggest that Rb and IRb could improve the osteoblast differentiation and alter the composition of matrix bone.

Our study also showed that Rb and IRb may play a pivotal role in the bone turnover by increasing BMP2 cytokine expression, the main driving force of *in vitro* osteoblast differentiation [47] and at the same time stimulating the osteoclastogenesis by maintaining high RANKL/OPG ratio [48]. RANKL (receptor Activators of NF-kB Ligand) expressed on osteoblastic cell membranes stimulates osteoclastogenesis, while osteoprotegerin (OPG) secreted by osteoblasts inhibits osteoclastogenesis [49, 50]. High MMP2 and MMP9 activities in cells treated with flavins indicate that the bone remodeling was also stimulated. The changing in the profile of MMPs has been attributed to the maturation of collagenous extracellular matrix during the differentiation osteoblastic process [51].

The capacity of flavins to trigger osteoblasts differentiation was also reinforced by upregulation of connexin 43, down regulation of caveolin-1 and negative modulation of Notch cascade. Connexin 43 expression is one of the major proteins responsible for maintaining intercellular communication between osteoblasts. Our data are in agreement with the works that showed an increase of gap junction-mediated intercellular coupling during the maturation of osteoprogenitor cells [52, 53]. On the other hand, caveolin-1 is a protein described to maintain a less differentiated state of osteoblast progenitor cells [54]. Notch signaling has been reported to maintain bone marrow mesenchymal progenitors by suppressing osteoblast differentiation [55] and in osteoblast can be one of the causes of osteopenia and impairs osteoblastogenesis [56].

5. Conclusion

In conclusion, this study brought out strong evidences that Rb and IRb generates an osteogenic microenvironment through modulating different mediators of signaling pathways related to survival, apoptosis, differentiation, osteoclastogenesis and osteoblastogenesis processes. Furthermore, in the absence of osteogenic inductors, Rb and IRb negatively regulated the protein expression associated with the suppression of osteoblast differentiation. In summary, this study pointed out the potential application of Rb and its photoproducts in osteoblasts phenotype development and consequently an alternative therapeutic adjuvant of osteoporosis.

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Capítulo 4

EFEITO ADITIVO DA RIBOFLAVINA E SEUS FOTOPRODUTOS DURANTE A DIFERENCIAÇÃO DE PRÉ-OSTEOBLASTOS MC3T3-E1 INDUZIDA POR ÁCIDO ASCÓRBICO E β-GLIGEROFOSFATO

"ADDITIVE EFFECT OF RIBOFLAVIN AND ITS PHOTOPRODUCTS DURING THE ASCORBATE AND β-GLYCEROPHOSPHATE-INDUCED PREOSTEOBLAST DIFFERENTIATION OF MC3T3-E1"

Additive effect of riboflavin and its photoproducts during the ascorbate and β-glycerophosphate-induced preosteoblast differentiation of MC3T3-E1

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ABSTRACT Since photoderivatives of riboflavin have been shown to modulate the proliferation rate and survival of cancer cells, we decided to examine the influence of these compounds in osteoblast cells during the ascorbic acid and β-glycerophosphate-induced differentiation process. In this study, we found that riboflavin and its photoproducts significantly affected the osteoblast proliferation, alkaline phosphatase activity, collagen biosynthesis, osteopontin and osteocalcin mRNA expression, metalloproteinases activities (MMP-2 and MMP-9) and the expression of osteoclastogenesis factors (RANKL and OPG). We also showed that the effects of flavins in osteoblasts cells were independent on flavins antioxidant property. The biological activity of the combination of osteogenic medium with riboflavin and its photoderivatives was associated with the activation of different signaling pathways (AKT, FAK, CaMKII), caspases -8, -9 and -3, and up-regulation of expression and/or stabilization of osteoblastic transcription factors (Runx2 and β-catenin). Our findings provide evidences of the potential of flavins as adjuvant to improve bone metabolism.

Keywords: Riboflavin; Osteoblast; Proliferation; Extracellular matrix; Differentiation.

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1. Introduction

Osteoporosis is described as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (No authors listed, 1993). This pathology is influenced by genetic and environmental factors such as diet and lifestyle. Therefore it is important to develop strategies and recommendations not only for the treatment but also for the prevention of osteoporosis. Researchers became highly interested in nutrients, which have a potential to prevent or minimize the risk to develop osteoporosis (Weber, 1999; Cashman, 2007; Trzeciakiewicz et al., 2009).

Riboflavin is the precursor for essential flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FAD and FMN are the prosthetic groups of numerous enzymes that catalyze the various electron transfer reactions that occur in energy-producing, biosynthetic, and detoxifying and electron-scavenging pathways (McCormick et al., 1989). Besides being important component of coenzymes, other biological properties of riboflavin have been described. Since dietary B vitamins can influence circulating homocysteine levels, some authors have examined the relationship among methylenetetrahydrofolate reductase (MTHFR) polymorphism and B complex vitamins and have been determined the raises of possibility that riboflavin intake and MTHFR genotype might interact to regulate the bone mineral density (Macdonald et al., 2004; Yazdanpanah et al., 2007; Yazdanpanah et al., 2008).

We have recently provided evidence that photoderivatives of riboflavin have strong activity in haematological malignancy (de Souza et al., 2006) and human prostate cancer cells (de Souza Queiroz et al., 2007). Considering that chemical structure of compounds is related to their biological activity and thus activation of different signaling pathways could be involved in their mode of action, we investigated whether riboflavin (Rb) or its photoproducts (IRb) combined with classical osteogenic inductors could reveals an additive or synergistic effect on bone component during the differentiation process. Therefore, we used the MC3T3-E1 pre-osteoblastic cell line, a well-accepted model of osteogenesis *in vitro* characterized for the induction of specific genes associated with the osteoblastic phenotype, including type I collagen, alkaline phosphatase, osteopontin and osteocalcin during the ascorbic acid and β-glycerophosphate-induced differentiation process (Sudo et al., 1983; Quarles et al., 1992; Franceschi & Iyer, 1992; Fratzl-Zelman et al., 1998).

The additive effect of riboflavin and its photoproducts during the ascorbate and β-glycerophosphate-induced osteoblast differentiation of MC3T3-E1 were characterized by increase of collagens synthesis and alkaline phosphatase activity coupled to the down-regulation of proliferation and multiples pathways signaling activation. In summary, this study provides clues to alternative therapeutic approaches against osteoporosis and show that photoproducts of riboflavin warrants further new investigations.

2. Materials and methods

2.1. Materials and Reagents

Ascorbic acid (AA), β-glycerophosphate (β-GP), riboflavin (Rb), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N,N-dimethylaniline (MDA), 4-aminoantipyrine (4-AAP), 1-chloro-2,4-dinitrobenzene (CDNB), 2, 6-colorimetric reagent dichloroindophenol (DCIP), N-methyl-2-phenylindole (MPO), 5, 5′-dithiobis (2-nitrobenzoate) (DTNB), p-nitrophenylphosphate, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red), were purchased from Sigma-Aldrich

(Sigma Chemical Co., St. Louis, MO). MC3T3-E1 preosteoblast cells were from American Type Culture Collection (ATCC, Rockville, MD). Polyclonal antibodies against phospho-AKT Thr308, phospho-CaMKII Thr286, phospho-FAK Tyr577, phospho-GSK3β, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-PP2A Tyr307, Bcl2, Bax, OPG, RANKL, Runx2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP-1 (p116/p25), β-catenin were from Upstate Biotechnology, Inc. (Lake Placid, NY). Caspases 3, 8 and 9 Colorimetric Assay Kits were obtained from R&D Systems (Minneapolis, MN). All other chemicals and reagents used in this study were of analytical grade.

2.2. Cell culture condition

MC3T3-E1 preosteoblast cells were routinely grown in modified alpha minimum essential medium (α-MEM) without AA, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics and incubated in a humidified atmosphere at 37 °C and at 5% CO_2. For all experiments, cells were plated at an initial density of 50,000 cells/cm^2. Differentiation was induced by adding osteogenic medium (OM) containing 50 μg/ml AA and 10 mM β-GP. All treatment conditions were performed after 24 h of cell attachment (day 0). After plating, cells were treated every three or four days.

2.3. Riboflavin irradiation

Solution of 250 μM Rb in α-MEM medium at the pH 7.44 (15 mL) was placed in a Petri dish and irradiated with UVC light ($\zeta_{máx}$=253.5 nm) for 30 min; the lamp was placed 40 cm from the Rb solution. Alpha-MEM medium was irradiated in the same conditions and used as a control. After irradiation the percentage of Rb and its photoproducts was the following, as determined by mass spectrometry: 79% of riboflavin, 6.2% of lumichrome, and 14.8% composed of formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin (de Souza Queiroz et al., 2007).

2.4. Treatment of cells with irradiated riboflavin

Cells were plated at an initial density of 50,000 cells/cm^2 in 96-well tissue-culture plates. All treatment conditions were performed after 24 h of cell attachment (day 0) and then treated with OM plus different concentrations of riboflavin (Rb) or irradiated riboflavin (IRb) (5–50 μM final concentrations) for 24 h. Cell viability was assessed by the MTT reduction, nucleic acid content and neutral red uptake assays.

2.5. Cell viability assays

2.5.1. Nucleic Acid Content

Cell number in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. (1991). Cells were washed twice with cold (PBS) and a soluble nucleotide pool was extracted with cold ethanol. Cell layers were then dissolved in 0.5 M NaOH at 37°C/1 h. Absorbance at 260 nm of the NaOH fraction was used as an indicative of cell number. Results are expressed as mean percentage of absorbance at 260 nm in treated wells compared with controls.

2.5.2. MTT reduction assay

Medium containing irradiated riboflavin was removed and 0.2 ml of MTT solution (0.5 mg MTT/ml of culture medium) was added to each well. After incubation for 4 h at 37 ºC, the medium was removed and the formazan released by solubilisation in 0.2 ml of ethanol. The plate was shaken for 5 min on a plate

shaker and the absorbance measured at 570 nm (Mosmann, 1983).

2.5.3. Neutral Red Uptake

NRU assay was performed according to the method of Borenfreund and Puerner (Borenfreund and Borrero, 1984). After 4 h of incubation with serum-free medium containing 50 µg neutral red/ml, the cells were washed quickly with PBS and then 0.1 ml of a solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After shaking on a microtitre plate shaker the absorbance was read at 540 nm.

2.6. Determination of Cell Cycle and Apoptotic Cell Death with Fluorescence-activated Cell Sorter (FACS)

MC3T3-E1 cells were plated on 6 cm diameter dishes at a density of 50,000 cells/cm^2 and cultured in the presence of 10% FBS medium for 24 h. For serum deprivation experiments cells were washed three times in PBS and cultured in serum free αMEM. After 24 h of serum starvation, cells were treated with OM plus 5 µM of Rb or IRb for 24 h. After this period the cells were harvested and deattached by trypsinization. Cells were washed with cold PBS and resuspended in paraformaldehyde 2% in PBS at 4 °C. Subsequently, 1 ml of propidium iodide solution was added and maintained at 4ºC for 15 min. The analysis was performed in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA,USA). It was analyzed 10,000 events per sample. DNA content was evaluated using a FL2 detector in a linear scale. To eliminate cell aggregates, the cell population to be analyzed was selected from a bivariate histogram showing the area (FL2A) *versus* the width (FL2W) of the signal FL2. The analysis of cell percentage in the different phases of the cell cycle (Sub-G_1, G_0/G_1, S/M) was performed using the CellQuest 3.4 software (BD Biosciences, San Jose, CA, USA).

2.7. Osteoblast differentiation markers assays

2.7.1. Cell proliferation assay

Cell number was determined by haemocytometer counts after trypsinization and represented as the means ± standard deviation (SD) of triplicates.

2.7.2. Alkaline phosphatase activity assay

Alkaline phosphatase activity was measured in the total cellular lysate after sonication in buffer containing 1 mM Tris (pH 8.8), 0.5% Triton X-100, 10 mM Mg^{2+} and 5 mM p-nitrophenylphosphate as a substrate. The reaction was stopped with 1M NaOH and the absorbance measured at 405 nm (Simão et al., 2007).

2.7.3. Collagen staining and semi-quantitative analysis

Pre-osteoblast cell differentiation into matrix-producing osteoblasts was assessed by quantification of collagen production using Picrosirius Red staining as previously described (Tullberg-Reinert and Jundt, 1999).

2.7.4. mRNA expression of matrix proteins and osteoblast markers

Total RNA was isolated from cells using Trizol reagent (Sigma-Aldrich Zwijndrecht, Netherlands) according to manufacturer's instructions. Reverse transcription was performed on 5 ng of total RNA using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 µl. Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Invitrogen, Breda, Netherlands) on the Biometra PCR system. PCR primers for mice were selected from multiple exons: osteopontin

(NM_009263.1), 5′-tctgatgagaccgtcactgc-3′ and 3′-cacccgagagtgtggaaagt-5′; osteocalcin (NM_007541.2), 5′-gcgctctgtctctctgacct-3′ and 3′-gccggagtctgttcactacc-5′; β-actin (NM_007393), 5′-tctcttccagccctctttca-3′ and 3′-atggtggtgcctccagatag-5′. The cycling program was 94°C for 2 min, 58°C for 60 seconds, 72°C for 60 seconds for the first cycle and 94°C for 30 sec, 58°C for 60 seconds, 72°C for 60 seconds for 30 cycles. PCR products were analyzed by 3% agarose gel electrophoresis and stained with ethidium bromide. The level of mRNA expression is expressed relative to the β-actin level. All experiments were performed in triplicate.

2.8. Zymographic analysis

Proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by de Souza et al. (2000). After the treatment the culture medium was collected and stored at -20 ºC in the presence of 1 mM PMSF (phenyl-methylsulphonyl fluoride-serine-protease enzyme inhibitor). Samples were diluted in non-reducing buffer (0.1 M Tris–HCl, pH 6.8, 20% glycerol, 1% SDS and 0.001% bromophenol blue). Volume of the samples loaded was proportional of the sample protein and resolved by SDS–polyacrylamide gel (10%) and 4% gelatin. Protein renaturation was performed in 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris–HCl and 10 mM CaCl2 (pH 7.4) at 37 ºC for 18 h. Gels were stained with 0.5% Coomassie blue G 250 for 30 min and then washed in a 30% methanol and 10% glacial acetic acid solution. Image analysis was performed on a PC computer using ScionImage software, freely available on the internet from Scion Corporation at http://www.scioncorp.com.

2.9. Redox status Analysis

After a further 7 days, to prepare cell cultures for assays, the cells were rinsed three times with cold, sterile, phosphate-buffered saline and harvested. Following centrifugation of the cells at 4°C and 1,500 rpm for 5 min, the supernatant was discarded. Cell pellet was resuspended in 400µl of ice-cold 10mM phosphate buffer (pH 7.0) and sonicated in an ice bath for 15s. After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatant was removed for assays of antioxidant enzymes, as well as malondialdehyde (MDA), total GSH and protein quantification. CAT activity was measured by H_2O_2 decomposition and monitored 30 minutes in absorbance at 230 nm of a reaction medium containing 10 mM H_2O_2, 50 mM sodium phosphate buffer (pH 7.0), and the enzyme sample (Maehly and Chance, 1957). Total SOD activity was measured by the coupled reaction of DMA with 4-AAP and absorbance determined at 554 nm (Tang et al., 2002). GST activity based on the conjugation of CDNB with glutathione and the activity determined at 340 nm as described by Habig et al. (1974). GPX activity was measured at 620 nm, employing DCIP and hydrogen peroxide as the substrate by method of Hawkes & Craig (1990). Lipid peroxidation as described by Gomes-Marcondes and Tisdale (2002). MDA was determined using MPO as the substrate. The resulting absorbance was measured at 570 nm and the results were expressed in nanomoles per milligram protein. For total glutathione content was used standard curves generated with known amounts of glutathione and DTNB as substrate (Ellman, 1959). Absorbance was measured at 412 nm and values expressed as nmoles/µg protein. All of the antioxidant enzyme activities were expressed as ΔOD/minutes/µg protein. The absorbance was measured an ELISA plate reader (Athos Labtech, Sussex, UK).

2.10. Western blotting analysis

Following treatment of cells for 7 days, the medium was aspirated and the cells were washed with cold physiological solution. Cells were then incubated in 200 µl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride)) over ice for 30 min. Protein extracts were cleared by centrifugation and protein concentrations were determined using Lowry method (1951). An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples which were subsequently boiled for 10 min. Cell extracts, corresponding to 75 µg of protein, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (1%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4 ºC with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 h. Bands detection was made using enhanced chemiluminescence ECL. Western blots represent three independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning the film (data are presented as the protein to β-actin ratio).

2.11. Caspases 3, 8 and 9 activity assays

Caspases activities were determined by the measurement at 405 nm of p nitroaniline (pNA) released from the cleavage of Ac-DEVD-pNA, IETD-pNA and LEHD-pNA as substrates of caspases 3, 8 and 9, respectively. Enzyme activities were expressed in pmol/min and the extinction coefficient of pNA was 10,000 $M^{-1}cm^{-1}$.

2.12. Statistical analysis

Results are expressed as mean ± S.D. with at least three replicates in each group. Differences were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P values < 0.05 were considered significant. All data were analyzed using GraphPad Prism Software, Version 4.0.

3. Results

3.1. Interaction of osteogenic medium with Riboflavin and Irradiated Riboflavin do not affect osteoblast cells viability

To determine the possible toxic effects between the interaction of osteogenic medium (OM – 50 µg/mL of ascorbic acid and 10 mM of β-glycerophosphate) with 50 µM Rb (OM+Rb) and the photoproducts of 50 µM IRb (OM+IRb) the preosteoblasts cells were treated for 24 h and the effect on cell viability was determined employing the MTT reduction, neutral red uptake and nucleic acid content assays. IC_{50} values were not reached in both treatment conditions; however differential sensitivities between the NR and MTT assays were detected from the concentration of 30 µM of IRb (Figure 1).

3.2. Riboflavin and Irradiated Riboflavin intensified G_0/G_1 arrest in preosteoblast cells

Osteoblastic differentiation is tightly linked to cell cycle regulation; therefore to determine whether the treatment with Rb or IRb might affect the cell cycle progression associated with the differentiation process induced by OM, we conducted FACS cell-cycle

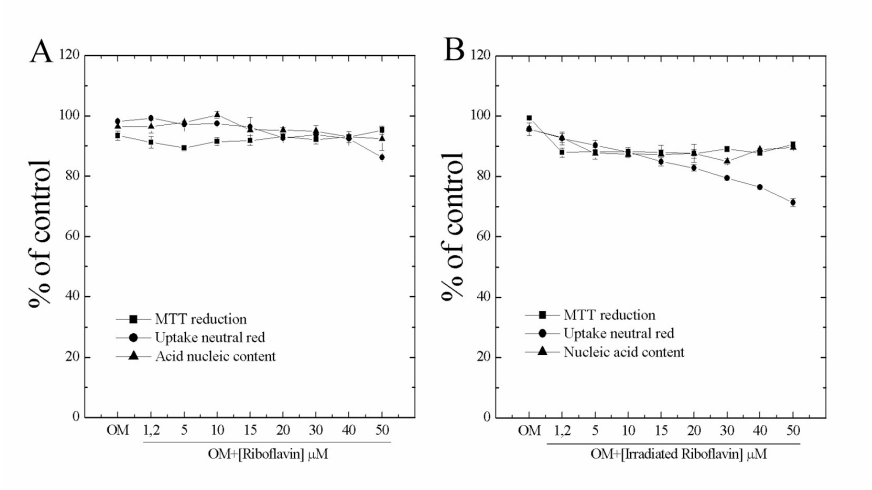

Fig. 1. Interaction of Riboflavin (Rb) and Irradiated Riboflavin (IRb) with osteogenic medium (OM – 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) do not affect preosteoblast cells viability. Cells were treated with different concentrations of Rb (A) or IRb (B). Cell viability was evaluated after 24 h. The MTT reductions, incorporation of neutral red and acid nucleic content in the OM group were considered as 100%. The experiment was performed in a 96 wells plate and results represent the means ± D.S. of 3 experiments run in triplicate.

analysis. The results showed that OM+Rb and OM+IRb attenuate cell cycle progression through the G_0/G_1 arrest in MC3T3-E1 cells as early as 24 h after the treatment ($P < 0.05$) (Table 1). The sub-G_1 peak, indicative of the apoptotic fraction due to fragmented DNA content, showed no significant difference in both treatments (Table 1).

Table 1. Evaluation of cell cycle progression in preosteoblastic MC3T3-E1 after addition of riboflavin and irradiated riboflavin at osteogenic medium

Cells	Sub-G_1	G_0/G_1 phase	Synthesis phase/Mitosis
OM	1.67 ± 0.66	48.56 ± 1.31	49,785±1,96
OM+Rb	0.95 ± 0.10	55.00 ± 0.91*	44,055±1,02
OM+IRb	2.53 ± 1.10	56.61 ± 0.27*	40,865±0,82*

After 24 h of serum starvation cells were treated with osteogenic medium (OM 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) plus 5 μM riboflavin (OM+Rb) or 5 μM irradiated riboflavin (OM+IRb) for 24 hours. Cells were stained with propidium iodide and the DNA content was analyzed by flow cytometry. Values of the percentages of MC3T3-E1 remaining in the Sub-G1, G0/G1 phase and Synthesis/Mitosis phase are shown on table. Results represent the means ± SEM of three independet experiments. * P<0.05 vs. OM and # P<0.05 vs. OM+Rb.

3.3. Effects of Riboflavin and Irradiated Riboflavin on osteoblastic differentiation markers and osteoclast-inducing factors

MC3T3-E1 cells, cultured in the presence of serum and ascorbate, express ALP and produce an extensive collagenous extracellular matrix that can be mineralized by the addition of β-glycerophosphate. We examined the effect of Rb and IRb in the proliferation and differentiation of preosteoblasts during 14 days in culture after treatment with osteogenic medium. Figure 2A shows the growth curve of MC3T3-E1 cells treated with OM plus 5 μM of Rb or IRb. At day 7, increase in cell proliferation occurred at all treatment, however proliferation rates of OM+Rb and OM+IRb groups were significantly lower compared to the OM group. After 7 days, all groups showed the reduction of total number of osteoblast and more intensely in the OM+Rb and OM+IRb (Fig. 2A). The decrease of total number of osteoblast coincided with an increase in the ALP, which was higher in the OM+IRb ($P < 0.05$) (Fig. 2B). To investigate the induction of collagen production, we utilized Picrosirius Red staining. We found a slightly increase of collagen deposition after 14 days of OM+Rb and OM+IRb treatments (Fig. 2C). Osteoblasts secrete complex extracellular matrix (ECM) containing

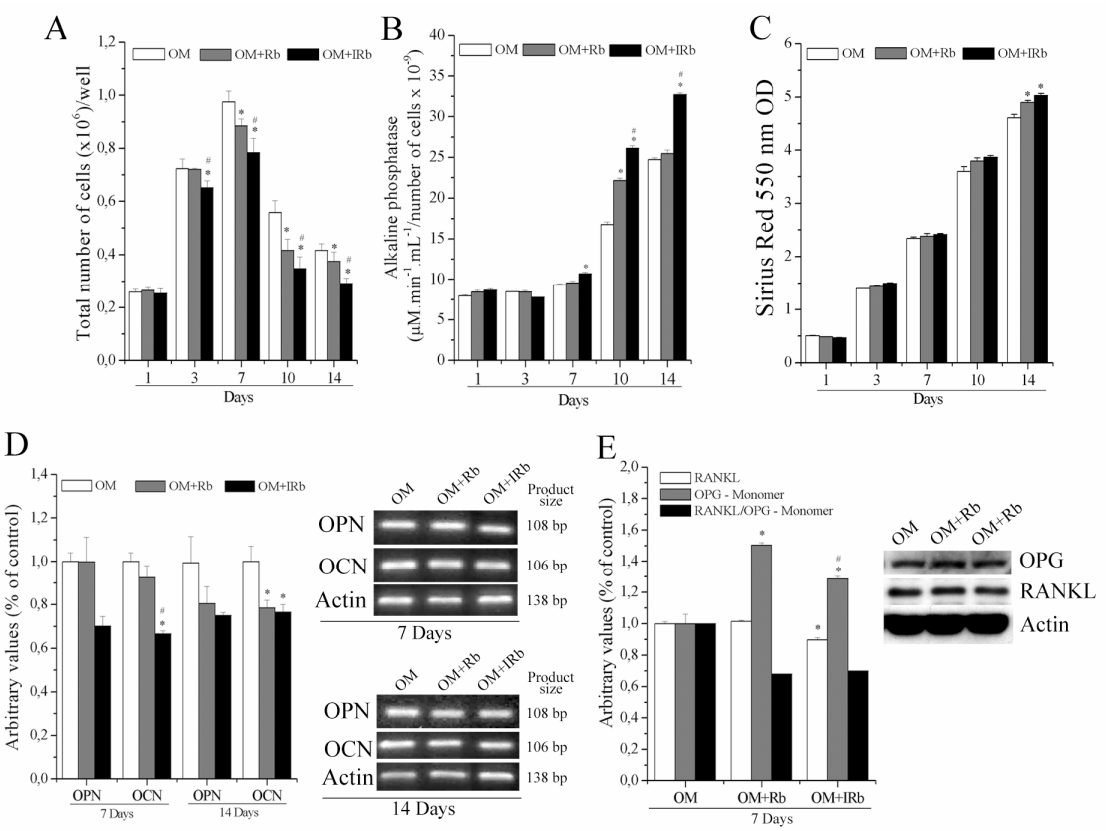

Fig. 2. Time course response of MC3T3-E1 cells at association of OM to Rb and IRb. (A) Cell number was assessed by direct counting after the stated time of culture; (B) Alkaline phosphatase activity during the treatment was measured using p-nitrophenylphosphate as a substrate; (C) Type I collagen production by the culture as assessed by Picrosirius staining; (D) mRNA expression of OCN and OPN in the cultures at the 7 days and 14 days as analyzed by RT-PCR; (E) Expression of osteoclast-inducing factor (RANKL/OPG). MC3T3-E1 cells were treated with OM (50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) plus 5 μM of Rb or IRb. All treatment conditions were performed after 24 h of cell attachment (day 0). Measurements were taken during 14 days of treatment. Each data value represents the mean result of triplicate or quadruplicate samples from a single representative trial; error bars represent the standard deviation of these samples. Experiments were repeated at least twice. * P<0.05 vs. OM and # P<0.05 vs. OM+Rb.

noncollagenous proteins. Osteopontin (OPN) and osteocalcin (OCN) are noncollagenous proteins considered early and late markers of osteoblastic differentiation, respectively. In this study, we investigated whether the OM+Rb and OM+IRb affects the mRNA expression of OCN and OPN after 7 and 14 days of treatment. A significant decrease in the OCN expression was detected after 7 and 14 days of OM+IRb treatment ($P < 0.05$), while for OM+Rb group this reduction was considered significant after 14 day ($P < 0.05$) (Fig. 2D). In our experiments, both treatment also decreased OPN expression after 7 and 14 days, but this alterations were not statistically significant (Fig. 2D). Homeostatic bone remodeling requires coordinated integration of biological signals between osteoblast and osteoclast. To investigate potential alterations in the modulation of bone metabolism after OM+Rb and OM+IRb we analyzed two factors supplied by osteoblast lineage cells, which are of critical importance for osteoclastogenesis: RANKL and osteoprotegerin (OPG). In this study, the reduction of the RANKL/OPG ration was driven by slightly decreased of RANKL expression and the significant increase of OPG expression in the OM+IRb ($P < 0.05$), while in the OM+Rb treatment we observed only the increase of OPG expression ($P < 0.05$) (Fig. 2E).

3.4. MMP2 and MMP9 activities are modulated by association of Rb or IRb and osteogenic medium

Matrix metalloproteinases are key mediators in extracellular matrix remodelling and primarily implicated in bone growth. Increased MMP expression can occur during injury, inflammation, cellular transformation, and oxidative stress. We examined whether the combination of osteogenic medium with riboflavin and its photoproducts could change a degradation of extracellular matrix due to the activity of matrix metalloproteinases (MMPs). Effect of OM+Rb and OM+IRb in metalloproteinases 2 and 9 activities was analyzed at 7 and 14 days after treatment of MC3T3 cells. MMP-9 activity showed an increase in the OM+Rb after both periods compared to the OM, while any change was observed in the OM+IRb group ($P < 0.05$) (Fig. 3), in contrast the MMP-2 activity decreased in the OM+Rb and OM+IRb groups at 7 and 14 days after the treatment ($P < 0.05$), however these changes were higher in the OM+IRb group (Fig. 3).

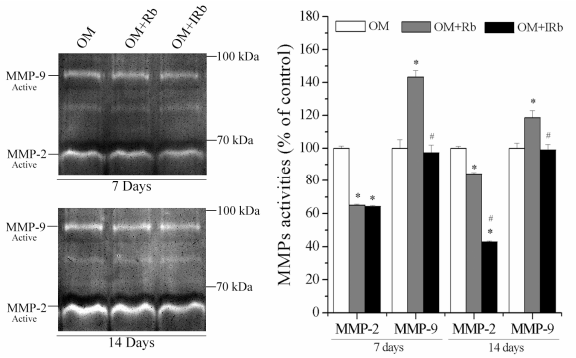

Fig. 3. The effect of OM+Rb and OM+IRb on activities of MMP-2 and MMP-9. Representative zymography of conditioned media from MC3T3-E1 cells treated with osteogenic medium (OM - 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) plus 5 μM of Rb or IRb for 7 and 14 days. The loading medium volume on the gel was normalized by the protein samples. Shown is the mean of three independent experiments ± S.D. * P<0.05 vs. OM and # P<0.05 vs. OM+Rb.

3.5. Combination of Rb or IRb with osteogenic medium actives multiple signaling pathways

Cellular response to extracellular stimulus and many other signaling molecules involves the activation of several signal-transduction pathways. Signal transduction from the extracellular environment to the nucleus requires the formation of many molecular complexes in which multiple proteins are assembled to directly or indirectly induce molecular events responsible for proliferation, differentiation and osteoblast death. In this context we studied the expression and activity of kinases and phosphatases involved in the osteoblast differentiation 7 days after the OM+Rb and OM+IRb treatment. Multiples signaling mediators were up-regulated in response to the both treatments (Fig. 4). We found that OM+Rb and OM+IRb treatments increased the phosphorylation/activation of AKT and consequently the phosphorylation/inhibitory of GSK3β (Fig. 4A). Furthermore, we also observed the increase of phosphorylation/inhibitory of PP2A, a negative regulator of survival pathway (Fig. 4A). The diversity of signaling pathways up-regulated was also demonstrated by phosphorylation/activation of focal adhesion kinase (FAK), calcium/calmodulin-dependent protein kinase II (CaMKII) and increase of cAMP-dependent protein kinase (PKA) expression (Fig. 4A). The findings about the involvement of proapoptotic mediators in anabolic stimulation during the osteogenesis are providing new clues to this process. Therefore, we investigated the impact of OM+Rb and OM+IRb in the expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins, as well as the activation of caspases-8, caspases-9 and caspases-3. Our results showed that flavins did not cause significant alteration in the Bax/Bcl-2 ratio (Fig. 4B). Despite this result, we observed significant activation of extrinsic and intrinsic

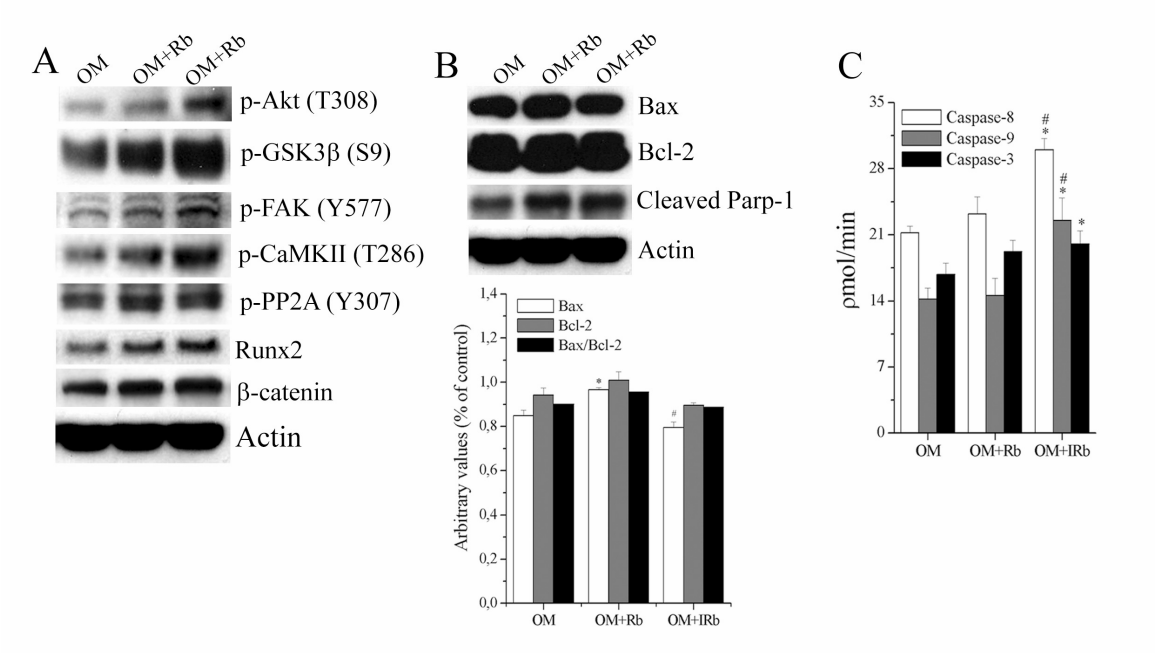

Fig. 4. OM+Rb and OM+IRb activities multiply signaling pathways and induce changes in key apoptotic regulators. MC3T3-E1 cells were treated for 7 days with OM plus 5 μM of Rb or IRb. (A) Both treatments promoted the phosphorylation/activation AKT, FAK, CaMKII, down-regulation of PP2A phosphatase and increase of osteoblastic transcriptions factors expression (β-catenin and Runx2). (B) Western blotting analysis of Bcl-2, Bax and cleaved PARP-1 levels and quantitative analysis of Bax/Bcl-2. Quantification of immunoband intensities from Western blotting analysis was determined by densitometric scannig. Data were expressed as Bax/Bcl-2 ratio. (C) Colorimetric assay was performed to determine the activation of caspase-8, caspase-9 and caspase-3. Detection of proteins was performed by Western blot as described in materials and methods. Results are representative of 3 different experiments. Values are expressed as means ± SD. * P<0.05 vs. OM and # P<0.05 vs. OM+Rb.

apoptotic pathways in the presence of OM+IRb ($P < 0.05$) as indicated by caspase-8, caspase-9 and caspase-3 activation (Fig. 4C), which was confirmed by increasing the PARP-1 cleavage, a substrate of caspases-3 (Fig. 4B). All changes observed in the signal transduction after the treatments favored the stability and up-regulation of osteoblastic transcriptions factors (β-catenin and Runx2) (Fig. 4A).

3.6. Combination of Rb or IRb and osteogenic medium does not change the redox status in osteoblast cells

Antioxidant and pro-oxidant properties of ascorbic acid and riboflavin have been well established by both *in vitro* and *in vivo* studies, but there are no reports about the redox status after the association of ascorbic acid and the photoproducts of riboflavin in osteoblast cell culture. To investigate the alterations of scavenging system enzymes and biomarkers of oxidative stress in osteoblast cells after 7 days of OM, OM+Rb and OM+IRb treatment, we measured the levels of MDA and total GSH, SOD, CAT, GST and GPX activities. After 7 days of treatment, we did not detect significant difference of MDA level and total GSH content and antioxidant enzymes (SOD, GPX and CAT) (Table 2). However, GST activity increased in the OM+Rb and OM+IRb treatments ($P < 0.05$).

4. Discussion

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass which results in a markedly increased risk for traumatic fractures (Ferreti et al., 2003). This disease is a worldwide health problem with a high prevalence. Agents for the treatment of osteoporosis are classified as either antiresorptive or anabolic; both therapies to osteoporosis modify the bone remodeling and have limitations. Research has provided insights into dietary

components that may to minimize the risk to develop osteoporosis by optimization of bone health and stimulatory effect in the bone formation. In this context, alternative therapies have been investigated to improve the bone quality by changes in the composition of matrix extracellular.

Table 2. Total GSH and MDA levels and SOD, CAT, GST and GPX activities in preosteoblast cells after 7 days of treatment with osteogenic medium and plus 5 μM of riboflavin (OM+Rb) or irradiated riboflavin (OM+IRb)

Parameters	Experimental groups		
	OM	**OM+Rb**	**OM+IRb**
MDA	9.37 ± 0.78	11.15 ± 1.00	8.16 ± 1.53
Total GSH	1.43 ± 0.06	1.48 ± 0.05	1.45 ± 0.08
SOD	1.60 ± 0.08	1.70 ± 0.08	1.91 ± 0.08
CAT	0.15 ± 0.02	0.22 ± 0.03	0.25 ± 0.05
GST	5.06 ± 0.31	6.50 ± 0.25*	7.94 ± 0.42*,#
GPX	0.71 ± 0.04	0.63 ± 0.08	0.57 ± 0.08

Values are expressed as means ± SD.; n = 12 for each experimental group. (*) P<0.05 vs. OM and (#) P<0.05 vs. OM+Rb.
GSH, as nmole total GSH/μg of protein
MDA, as nmole MDA/μg of protein
SOD = superoxide dismutase (ΔOD/min/μg of protein)
CAT = catalase (ΔOD /min/μg of protein)
GST = glutathione S-transferase (ΔOD /min/μg of protein)
GPX = glutathione peroxidase (ΔOD /min/μg of protein)

Despite the considerations mentioned above, the molecular effect of high riboflavin concentrations in osteoblast cells during the differentiation process has not been described and discussed. In this study, we examined whether the combination of classical osteogenic inductors (ascorbic acid and β-glycerophosphate) with riboflavin and its photoproducts (formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin) could reveals an additive or synergistic effect on bone components during the osteoblastic differentiation. This work is the first investigation that provided clear evidence how riboflavin and irradiated riboflavin can directly modulate the osteoblast function during the differentiation of the preosteoblastic cell line and reported which cellular signaling pathways are potentially implicated. Using the nontransformed preosteoblastic cell line (MC3T3-E1), a well-accepted model of osteogenesis *in vitro* (Quarles et al., 1992), we found that riboflavin and its photoproducts significantly affected the cell proliferation, alkaline phosphatase activity, collagen biosynthesis, osteopontin and osteocalcin mRNA expression, metalloproteinases acitivities (MMP-2 and MMP-9) and the expression of osteoclastogenesis factors (RANKL and OPG). We also showed that the effects of both treatments in the preosteoblasts cells were independently of their antioxidant properties. The biological activity of the combination of osteogenic medium with riboflavin and its photoproducts was associated with the activation of different signaling pathways (AKT, FAK, CaMKII), increase of caspase activities (caspases-8, -9 and -3) and up-regulation in the expression and/or stabilization of osteoblastic transcription factors (Runx2 and β-catenin).

We have found that riboflavin and its photoproducts were non-cytotoxic when associated with the osteogenic medium (ascorbic acid and β-glycerophosphate). However, the results demonstrate that neutral red uptake assay was more sensitive than the MTT and nucleic acid content tests to detect subcellular changes induced by photoproducts of riboflavin. With this result, we selected the concentration of 5 μM of riboflavin and irradiated riboflavin (subtoxic concentration) for proceeding the next experiments.

It is known that macro- and micronutrients may enter into the cells and then interact with transcriptions factors which are known to influence target gene expression (Müller and Kerten, 2003). Considering the field of osteoblast development, we suggested that the biologic effects of riboflavin and its photoproducts were associated with the upregulation of Runx2 and β-catenin transcription factors which in turn

can active osteoblast-related genes. Our results support the evidence that the increase of collagens synthesis and alkaline phosphatase activity were functionally coupled to the down-regulation of proliferation of osteoblast. On the other hand, we observed that riboflavin and its photoproducts down-regulated the osteopontin and osteocalcin mRNA expression. Runx2 is a multifunctional transcription factor that induce the expression or activate the promoters of bone extracellular matrix (type I collagen, osteopontin, bone sialoprotein, and osteocalcin) (Komori, 2005), furthermore Runx2 is linked to cell cycle regulation during the osteoblastic differentiation exerting anti-proliferative effects (Galindo et al., 2005). However, the down regulation of osteocalcin gene expression, a late marker of mature osteoblast, suggests that riboflavin and its photoproducts promoted a delay in the osteoblast differentiation process in consequence of the Runx2 expression increase. The reduction of Runx2 expression is necessary for differentiation into mature osteoblasts (Komori, 2005) and the overexpression of type II Runx2 is known for severely reduces osteocalcin expression in osteoblasts (Liu et al., 2001). Osteopontin, one of the bone matrix proteins, plays an important role in the attachment of osteoclasts to bone matrix, the down regulation of osteopontin have been described to affect the osteoclast attachment in bone cells cultured with Drynariae Rhizoma extracts (Sun et al., 2002), therefore this change could be considered other potential effect of riboflavin and its photoproducts.

Our study demonstrates that riboflavin and its photoproducts could affect bone remodeling at the cellular level, by decreasing the ratio RANKL/OPG, especially due to the increase in OPG, which is a potent inhibitor of osteoclastogenesis and secreted by osteoblastic stromal cells (Simonet et al., 1997; Yasuda et al., 1998; Shalhoub et al., 1999). OPG acts as the soluble decoy receptor of RANKL and exerts its effects by binding to and antagonizing RANKL actions. The expression of OPG has been shown to be regulated via stimulation of Wnt/β-catenin signaling, which increases the expression of osteoprotegerin (Glass et al., 2005).

Matrix metalloproteinases (MMPs) are capable to hydrolyze numerous ECM components and play essential roles in tissue remodeling under various physiological and pathological conditions. Changes in the MMP profiles in MC3T3-E1 cells are attributed to the maturation of collagenous extracelular matrix (ECM) induced by ascorbate-2-phosphate (Mizutani et al., 2001). Our data suggest that the combination of osteogenic medium with riboflavin and its photoproducts showed potential adverse effect in the extracellular matrix degradation by decreasing MMP-2 activity, however this effect could be compensated by MMP-9 increasing observed in the OM+Rb. Despite this attenuation in the metalloproteinases activity, this alteration did not compromise the collagen extracellular matrix accumulation and the rise in alkaline phosphatase activity induced by OM+Rb.

In this study, to clarify the mechanism of Rb and IRb, we studied AKT, GSK3β, FAK and CaMKII kinases that are known to play crucial role during the osteoblast differentiation. Preosteoblasts cells response towards riboflavin and its photoproducts treatment is based on the activation of AKT kinase and subsequent inhibition of GSK3β kinase via phosphorylation of Ser 9. AKT/PKB is a serine/threonine kinase that shares sequence homology with PKC and PKA, which mediates intracellular signaling by extracellular agonist and plays a crucial role in cellular functions such as proliferation and survival in a variety of cells (Coffer et al., 1998). Noda et al. (2005) reported that PI3K/AKT plays role in the IGF-I stimulated alkaline phosphatase in osteoblasts cells (MC3T3-E1). Recent work indicates that inhibition of GSK3 increases bone formation,

density, and strength in an ovariectomized rat model (Kulkarni et al., 2006). GSK3β is important during the osteoblastogenesis (Krishnan et al., 2006) and is involved in many biological events, including structure, gene expression, mobility, and apoptosis (Doble and Woodgett, 2003; Jope et al., 2007). Our results suggest that GSK3β repression contributed to stabilization and activation of Wnt/β-catenin signaling during the treatments. Protein phosphatase 2A (PP2A) has long been known to negatively regulate AKT activity (Kuo et al., 2008). In contrast to those findings reported by our group in tumor cells (Souza et al., 2006; de Souza et al., 2007), preosteoblasts displayed inhibition of PP2A after combination of Rb or IRb with osteogenic medium. Another aspect observed between the combination of osteogenic medium and the riboflavin photoproducts was the possibility of changes in the intracellular Ca2+ levels, as indicated by an increase in the CaMKII autophosphorylation. Ca2+/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitously expressed serine/threonine kinase that is activated by Ca2+ and calmodulin. This kinase has been implicated as a key regulator of osteoblast differentiation promoting the stability and the osteoblastogenic transactivation activity of Dlx5 transcription factor (Seo et al., 2009) and regulating the signaling pathways that PTH-induce expression of osteoclastogenic inhibitors in MC3T3-E1 (Zheng et al., 2009). Collagen matrix synthesis is required for inducting the differentiation markers due the critical role of type I collagen in mediating the signaling cascade for expression of a mature osteoblast phenotype and mineralization of extracellular matrix (Lynch et al., 1995). Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase implicated in cell–matrix interaction that has been shown to be increasingly phosphorylated by adhesion to type I collagen in MC3T3-E1 cells to stimulate osteoblastic differentiation (Takeuchi et al., 1997). These observations suggest that the effect of riboflavin and its photoproducts in the collagen matrix guarantee the FAK activity, which also could contribute for inhibitory phosphorylation of GSK3 (Huang et al., 2002).

Another point explored in this study was the expression/activity of potential apoptotic effectors in cell differentiation. Activation of caspases is required for BMP-4 osteoblastic differentiation MC3T3-E1 (Mogi and Togari, 2003), while Miura et al. (2004) demonstrated that caspase-3 is crucial for the differentiation of bone marrow stromal stem cells by influencing TGF-beta/Smad2 pathway and cell cycle progression. Our results showed strong activation of caspases-3, -8 and -9, indicating that these proteases are required for photoproducts riboflavin effects in the preosteoblasts during the osteogenic differentiation.

Each cell is characterized by a particular redox status in different cellular compartments. Cellular redox status and its oscillation might define the cellular fate, such as differentiation (Schafer et al., 2001; Arrigo, 1999). Changes in the redox status of cells are thought to be responsible for modulating cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors. Therefore, in this study we evaluated whether the effects of Rb and IRb combinated with osteogenic medium could be related to the alteration of osteoblast cells redox status during the differentiation process. Although the antioxidant and pro-oxidant properties of ascorbic acid and riboflavin have been well established by both *in vitro* and *in vivo* studies, both treatments have failed to promote significant alterations of scavenging system enzymes and biomarkers of oxidative stress status to explain the effects observed during the experimental periods. However, our data also pointed out the existence of an adaptive mechanism associated with an increase of GST activity, following the treatment of

MO+IRb. GST catalyses the conjugation of GSH with peroxidised lipids and xenobiotic substrates in the detoxification cellular (Matés, 2000; Ray and Husain, 2002), therefore this change can be due to the photoproducts generated are markedly more hydrophobic as compared to the parent compound riboflavin, therefore the results suggest that in high concentration into cell the photoproducts might be metabolized as xenobiotic substrate by GST.

In conclusion, these findings suggest that the combination of classical osteogenic inductors (ascorbic acid and β-glycerophosphate) with riboflavin or its photoproducts revealed an additive effect on some bone components during the osteoblastic differentiation, moreover the results demonstrate the potential effect in the osteoclastogenesis processes by decreasing RANKL/OPG ratio. The possible mechanism of these effects was stimulation of Runx2 and β-catenin expression, while the increase of collagens synthesis and alkaline phosphatase activity were functionally coupled to the down-regulation of proliferation. In summary, this study provides clues to alternative therapeutic approaches against osteoporosis and shows that riboflavin photoproducts deserve further investigation, including preclinical and clinical studies.

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Discussão

Discussão

Nos presentes trabalhos foram abordados os seguintes aspectos a respeito da diferenciação de pré-osteoblastos induzidas por ácido ascórbico e β-glicerofosfato (AA/β-GP): aplicação da tecnologia de microarranjos de peptídeos para a análise das vias de sinalização celular e o perfil de enzimas antioxidantes no estágio inicial do processo de diferenciação de osteoblastos. Os resultados obtidos nesta primeira etapa serviram de suporte para coordenar a segunda etapa deste trabalho onde os pré-osteoblastos foram tratados com riboflavina e riboflavina irradiada. Desta forma considerações relacionadas à citotoxicidade e os efeitos sobre o processo de diferenciação também foram caracterizadas.

1 Novos aspectos da sinalização celular implicados na diferenciação de osteoblastos induzida por ácido ascórbico e β-glicerofosfato

Na literatura há muitas informações sobre as propriedades do ácido ascórbico como um regulador na biossíntese de colágeno (Geesin et al., 1991, Pinnell, 1985), enquanto a interação com β-glicerofosfato é descrita como indutor de diferenciação de osteoblastos (Quarles et al., 1992; Franceschi and Iyer, 1992). Entretanto, o preciso mecanismo molecular acerca das ações não foram ainda totalmente elucidadas. Portanto, este fato nos motivou a investigar as vias de transdução de sinal moduladas durante a fase inicial de diferenciação induzida por AA/β-GP utilizando microarranjos de peptídeos.

Neste estudo nós observamos que AA/β-GP modula diferentes quinases como também a atividade de caspases. Nós encontramos que a AA/β-GP suprimiu as cascatas de sinalização PI3K/AKT e Src/ERK 1/2, o que indica que a supressão de ambas as cascatas são essenciais para a fase inicial do processo de diferenciação. Consistente com nosso achados, a inibição da via

PI3K tem sido relacionado com o processo de diferenciação de melanoma B16 (Buscà et al., 1996), linhagem de leucemia promielocítica HL60 (Peiretti et al., 2001), linhagem de células miogênicas (Viñas et al., 2002) e acelera a diferenciação terminal de condrócitos (Kita et al., 2008). Por outro lado, PI3K/AKT é um dos executores mais importantes na sinalização de potentes fatores anabólicos ósseos. Por exemplo, fatores de crescimento tais como PTH e IGF-1 podem ativar a via PI3K que se propaga através da ativação da AKT (Yamamoto et al., 2007; Nakasaki et al., 2008), enquanto proteínas Wnt prolongam a sobrevida de osteoblastos e células osteoblásticas não-comprometidas ativando cascatas de sinalização Src/ERK e PI3K/AKT (Almeida et al., 2005).

Zambuzzi et al. (2008) tem apresentado a diminuição da atividade da Src durante a diferenciação de pré-osteoblastos induzida por AA/β-GP, assim como a regulação temporal das vias PI3K/AKT e ERK, entretanto, é importante mencionar que neste trabalho não foi incluso a condição sem AA/β-GP.

Em adição, nós também demonstramos uma expressiva modulação negativa das quinases PAK2 e Syk. Proteína quinase ativada-p21 (PAK) foi identificada como a primeira quinase ativada por meio das pequenas GTPases Rac/Cdc42 (Manser et al., 1994) e pode ser ativada em respostas a hiperosmolaridade, irradiação, luz UV e drogas terapêuticas que danificam o DNA tais como a cisplatina. Syk é uma proteína tirosina quinase não-receptora expressa de forma onipresente em células hematopoiéticas e também é expressa em células epiteliais, endoteliais (Kurosaki et al., 2000; Inatome et al., 2001) e células osteoblásticas (Rezzonico et al., 2002). Recentes estudos sugerem que as PAK2 e Syk atuam como reguladores *upstream* das vias JNK e p38 (Frost et al., 1996; Zhang et al., 1995). Miah et al., (2004) demonstraram que PAK2 e Syk cooperam positivamente para regularem repostas celulares ao estresse através da ativação da JNK sob condições hiperosmóticas. Syk é o principal efetor *upstream* da via PI3K/AKT e participa da

ativação da PI3K, a qual exerce uma significativa função na regulação na mobilidade de células cancerosas (Jiang et al., 2002; Mahabeleshwar & Kundu, 2003). Tem sido sugerido que Syk exerce uma função crítica na morfogênese, crescimento, migração e sobrevida (Inatome et al., 2001). Portanto, os efeitos do AA/β-GP na supressão da PI3K e JNK poderiam ser associados com as diminuições das atividades das quinases Syk e PAK2.

Em relação à quinase PKA, nós demonstramos um aumento na atividade e expressão desta quinase. Wu et al. (2007) relatou que o aumento da incorporação do AA e consequente promoção da diferenciação de células pré-osteoblásticas, esta associado com a fosforilação do transportador 2 de Vitamina C dependente de sódio (SVCT2) através da PKA.

Um outro ponto explorado neste estudo foi à expressão e atividade de efetores apoptóticos juntos ao processo de diferenciação. Apesar de cessar o crescimento celular e promover a ativação das caspases-8, -9 e -3, além do aumento da razão Bax/Bcl-2 e clivagem da PARP-1, apoptose não foi o processo dominante engatilhado pelo AA/β-GP. Ativações de caspases são requeridas para a diferenciação de pré-osteoblastos MC3T3-E1 após indução através da BMP4 (Mogi &Togari, 2003), enquanto Miura et al. (2004) demonstraram que a caspase-3 é crucial para a diferenciação de células tronco do estroma da medula óssea através da influência da via TGF-β/Smad2 e progressão do ciclo celular. Por outro lado, andrógenos estimulam a apoptose de osteoblastos e osteócitos através de um aumento da razão Bax/Bcl-2 mesmo diante de sinais anabólicos e sugere que a apoptose acentuada pode estar associada com a estimulação anabólica do crescimento do novo osso (Wiren et al., 2006). Em relação à PARP, Harnacke et al. (2005) demonstraram que a repressão da PARP-1 em células leucêmicas humanas TUR restaura a responsividade transcricional para a diferenciação e parada do ciclo celular.

A função do AA/β-GP na produção da matriz de colágeno e expressão de marcadores de diferenciação tem sido caracterizada, entretanto a implicação na expressão de osteoprotegerina

(OPG) e RANKL ainda não está clara. OPG é produzida pelos osteoblastos e outros tipo de células (Simonet et al., 1997). Perturbações extrínsecas ou intrínsecas deste balanço OPG/RANKL resultariam diretamente da perda de controle do remodelamento ósseo levando as doenças esqueléticas tais como a osteoporose (Horwood et al., 1998; Nagai & Sato, 1999). Tem sido sugerido que a razão entre os níveis de expressão de OPG e RANKL em células osteoblásticas é o fator chave na reabsorção óssea. Nós temos observado que a redução da razão RANKL/OPG na fase inicial do processo de diferenciação osteoblástico, o qual foi na sua maior parte refletida pelo aumento dos níveis de proteínas homodímeras da OPG que provêem um aumento na afinidade para o RANKL (Theoleyre et al., 2004). Portanto, a diferenciação osteoblástica induzida por AA/β-GP diminui a osteoclastogênese por promover a formação do homodímero de OPG que é requerido para o mecanismo de inibição da interação receptor RANK/RANKL.

2 Diferenciação de osteoblastos induzida por ácido ascórbico e β-glicerofosfato aumenta a suscetibilidade ao estresse oxidativo

Neste trabalho, nós observamos que no estágio inicial de diferenciação dos osteoblastos, as enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPX) e glutationa S-trasferase (GST) apresentaram atividades reduzidas. Estes achados sugerem que o estado de diferenciação poderia definir a sensibilidade aos danos proporcionados pelo estresse oxidativo através da redução da atividade de enzimas antioxidantes, as quais podem ser relevantes para doenças ósseas como osteoporose.

Outra observação importante feita por diferentes grupos de pesquisa tem indicado que as propriedades antioxidantes do AA não são os principais fatores contribuintes para o processo de diferenciação. Portanto, as mudanças no estado redox da célula através de outros tipos de

antioxidantes (enzimáticos ou não-enzimáticos) parecem ser mais relevantes para a ocorrência da diferenciação de osteoblastos (Chen et al., 2008; Fatokun et al. 2008). Sob este aspecto, Varadharaj et al. (2005) examinaram o efeito de diferentes concentrações de ácido ascórbico em células endoteliais de pulmão, altas concentrações de AA (1 mM) resultaram na reduzida viabilidade celular e alterações morfológicas; entretanto, nenhuma mudança ocorreu neste estudo onde uma dose inferior (0,3 mM) foi aplicada com o objetivo de induzir o estado de diferenciação.

A capacidade do ácido ascórbico em induzir a diferenciação tem sido detectada em outros tipos celulares. A diferenciação induzida por AA em células leucêmicas HL60 (Kang et al., 2003) e rediferenciação de células de hepatomas humanos (Zheng et al. 2002) foram associadas com o aumento da atividade da SOD, diminuição da atividade da CAT e aumento da H_2O_2 e inibição da proliferação.

Em nosso estudo, a observação da significante redução da atividade das enzimas SOD, CAT, GST e GPX poderiam explicar a acentuada sensibilidade ao H_2O_2 durante a diferenciação de osteoblastos induzidos por AA/β-GP demonstrada por Fatokun et al. (2008).

3 Caracterização dos efeitos biológicos da riboflavina e riboflavina irradiada em pré-osteoblastos

Osteoporose é uma doença esquelética caracterizada pela diminuição da massa óssea, o qual resulta em um marcado aumento no risco de fraturas atraumáticas (Ferreti et al., 2003). Esta patologia é influenciada por fatores ambientais tais como a dieta e estilo de vida. Portanto, é importante o desenvolvimento de estratégias e recomendações não somente para o tratamento, mas também para a prevenção da osteoporose. Agentes para o tratamento da osteoporose são classificados em anti-reabsortivos e anabólicos. Agente anti-reabsortivos funcionam através da

inibição da atividade dos osteoclastos e, portanto, reduzem a reabsorção óssea, enquanto os agentes anabólicos atuam através da estimulação da formação óssea (Deal, 2009). Cientistas têm demonstrado interesse em nutrientes, os quais possuem potencial para prevenir ou minimizar o risco do desenvolvimento da osteoporose (Weber, 2007; Cashman, 2007; Trzeciakiewicz et al., 2009). Recentes evidências têm demonstrado que espécies reativas de oxigênio (EROs) podem estar envolvidas na patogênese da doenças relacionadas com perdas ósseas. Reduzida formação óssea é associada com o aumento do estresse oxidativo em mulheres e homens idosos (Basu et al., 2001). Marcada diminuição de antioxidantes no plasma é encontrado em mulheres com osteoporose (Maggio et al., 2003). Pesquisadores indicam que o estado geral da saúde óssea é melhorado com prolongada suplementação de vitaminas antioxidantes, as quais podem ser usadas como tratamentos paliativos para osteoporose (Basu et al., 2001; Maggio et al., 2003). Um desafio para os pesquisadores é identificar novos compostos que contribuam para a manutenção da saúde óssea e, por consequência, caracterizar os mecanismos de ação dos mesmos.

Baixa disponibilidade de riboflavina proveniente de dietas aumenta o risco de fraturas pós-menopausa em mulheres homozigotas para o polimorfismo C677T do gene metilenotetraidrofolato redutase (MTHFR) (Yazdanpanah et al., 2007), enquanto o aumento de riboflavina e piridoxina incorporadas à dieta foi associado com o aumento da densidade óssea na região do pescoço do fêrmur (Yazdanpanah et al., 2007). A suplementação com piridoxina e certas vitaminas do complexo B (riboflavin, folato e cobalamina) durante a senilidade tem chamado atenção, uma vez que foi associada com a manutenção de baixos níveis de homocisteína (Nygård et al., 1998; Yazdanpanah et al., 2007; Yazdanpanah et al., 2008), a qual é considerada um novo fator de risco para as fraturas osteoporóticas relacionados ao envelhecimento, principalmente em mulheres homozigotas para o polimorfismo C677T do gene MTHR (Yazdanpanah et al., 2008).

Apesar das considerações mencionadas acima, o efeito molecular de altas concentrações de riboflavina em osteoblastos não foi até o momento descrito e discutido. Portanto, neste estudo os efeitos biológicos da riboflavina e seus fotoprodutos (formilmetilflavina, lumiflavina e 2-cetoriboflavina e 4-cetoriboflavina), foram avaliados usando células pré-osteoblásticas. A linhagem pré-osteoblastica MC3T3-E1 é considerada um modelo excelente de diferenciação celular que simula os eventos iniciais da osteoblastogênese (Quarles et al., 2002).

Este trabalho focou a análise dos efeitos biológicos da riboflavina e seus fotoprodutos em duas distintas condições. Na primeira condição os efeitos biológicos da riboflavina e seus fotoprodutos foram caracterizados durante a diferenciação de osteoblastos estimulada pelos fatores de crescimento provenientes do soro fetal bovino (SFB) em meio de cultura fresco sem ácido ascórbico. Na segunda condição os efeitos biológicos da riboflavina e seus fotoprodutos foram caracterizados durante a diferenciação de osteoblastos promovida por indutores clássicos, ácido ascórbico e β-glicerofosfato, além dos componentes provenientes do SFB.

Apesar do efeito citotóxico da riboflavina não-irradiada ser descrito em culturas de células leucêmicas (HL60) devido à formação do estado "triplet" a partir da atividade de peroxidases (Collins et al., 1987; Rojas & Silva, 1988), nós não observamos qualquer efeito adverso na viabilidade celular de pré-osteoblastos. Mesmo a riboflavina irradiada, que tem apresentado forte efeito citotóxico em células leucêmicas (Souza et al., 2006) e células de câncer de próstata (de Souza et al., 2007), não causou efeitos citotóxicos significativos em pré-osteoblastos. Este aspecto da citotoxicidade dos fotoprodutos entre diferentes linhagens celulares está relacionada com o aumento da hidrofobicidade de moléculas fotosensibilizadas e sua acentuada afinidade por tumores e outras células hiperproliferantes (Kessel, 1989).

Na primeira condição analisada riboflavina e seus fotoprodutos causaram a parada do ciclo celular na fase G0/G1 e suprimiram a atividade da quinase AKT, um mediador da

proliferação. Os efeitos biológicos foram caracterizados pelo aumento da expressão de osteocalcina, osteopontina e BMP-2, no entanto foi observada a atenuação da atividade da enzima fosfatase alcalina e do acúmulo de colágeno na matriz extracelular. Em adição, foram constatadas maiores atividade das metaloproteinases MMP-9 e MMP-2. Deve ser destacado, que a capacidade das flavinas em engatilhar a diferenciação de osteoblastos foi também reforçada através do aumento da expressão de conexina 43, diminuição da expressão da caveolina-1, supressão da sinalização Notch e alterações no sistema enzimático antioxidante.

Na segunda condição a riboflavina e seus produtos demonstraram interagir com os indutores clássicos da diferenciação (ácido ascórbico e β-glicerofosfato) ao promover efeitos aditivos em alguns marcadores de diferenciação osteoblástica. Nesta segunda condição nos deparamos com o aumento da atividade da enzima fosfatase alcalina e o discreto aumento da síntese e acúmulo de colágeno na matriz extracelular. Apesar destes efeitos sinérgicos, a interação com os indutores de diferenciação atenuou a expressão de proteínas características da matriz extracelular óssea, osteocalcina e osteopontina, além de diminuir a atividade da metaloproteinase MMP-2, envolvida com a degradação e remodelamento da matriz extracelular. Nós consideramos que estes efeitos foram independentes do sistema enzimático antioxidante, pois nenhuma mudança significativa foi constatada. A atividade biológica da combinação dos indutores osteogênicos com riboflavina e seu fotoderivados, ao contrário da primeira condição, foi associada com a ativação de diferentes vias de sinalização (AKT, FAK, CaMKII), caspases -8, -9 and -3, e o aumento da expressão e/ou estabilização de fatores osteoblásticos de transcrição (Runx2 and β-catenin).

Outra variante encontrada entre as condições estudadas foi o potencial da riboflavina e seus fotoprodutos em modularem a diferenciação de osteoblastos. Através da análise da expressão dos fatores RANKL e OPG e o estabelecimento da razão entre os mesmos, pode-se

determinar que, quando isoladas, riboflavina e seus fotoprodutos proporcionaram um aumento da relação RANKL/OPG, o qual favorece a diferenciação de osteoclastos, enquanto a interação riboflavina e seus fotoprodutos com os indutores osteogênicos promoveram uma diminuição desta relação, o que caracteriza o seu potencial em inibir a diferencição de osteoclastos.

Nos últimos anos diversos estudos têm descrito os efeitos de drogas, compostos sintéticos, nutrientes e extratos vegetais em linhagens osteogênicas. Nestes trabalhos a estrutura química dos compostos bem como sua capacidade de interação com outros compostos tem sido considerada e relacionada aos seus efeitos biológicos, pois isto implica na ativação de diferentes vias de sinalização e alteração na expressão de proteínas essenciais para o estabelecimento de funções celulares. No intuito de se definir um composto ideal capaz de induzir a proliferação e diferenciação de osteoblastos muitas vias de sinalização foram descritas e moduladas de maneiras distintas e isto reflete consequentemente em aspectos qualitativos e quantitativos nos marcadores de diferenciação óssea. Estas considerações, portanto justificam a diversidade dos efeitos biológicos proporcionados pela riboflavina e seu fotoprodutos durante o processo de diferenciação osteoblástica *in vitro*.

Conclusões

Conclusões

Os resultados deste trabalho sugerem o potencial de ação direta da riboflavina e seus fotoprodutos em células da linhagem osteogênica. A análise da função de pré-osteoblastos na presença de flavinas demonstrou que tais compostos atuam em diferentes níveis moleculares modulando diversas vias de sinalização celular relacionadas com a proliferação, diferenciação, remodelamento de matriz extracelular e sistemas antioxidantes (Figura 8) o que nos leva as seguintes conclusões:

1- O estágio inicial de diferenciação de pré-osteoblastos induzido por ácido ascórbico e β-glicerofosfato é caracterizado: a) supressão das vias de proliferação e sobrevivência Src/ERK e PI3K/AKT; b) ativação de efetores apoptóticos através do aumento da atividade de caspases; c) ativação da proteína tirosina fosfatase PP2-A; d) diminuição na atividade das enzimas antioxidantes superóxido dismustase, catalase, glutationa peroxidase e glutationa S-transferase;

2- Riboflavina e seus fotoprodutos geraram um microambiente osteogênico através da modulação de diferentes mediadores de vias de sinalização relacionadas com a sobrevivência (AKT/p70 S6K), apoptose (caspase-8 e -3), diferenciação (Notch-1, conexina 43 e caveolina-1) e fatores protéicos envolvidos com a osteoclastogênese (RANKL/OPG) e osteoblastogênese (BMP2);

3- A combinação de indutores osteogênicos clássicos (ácido ascórbico e β-glicerofosfato) com riboflavina e seu fotoprodutos revelaram um efeito aditivo na atividade de fosfatase alcalina, além de promover a diminuição da relação RANKL/OPG o que desfavoreceria a osteoclastogênese. Estes efeitos foram associadas com a ativação de diferentes vias de

sinalização (AKT, FAK, CaMKII), caspases-8, -9 e -3 e o aumento da expressão e/ou estabilização de fatores de transcrição osteoblásticos (Runx2 e β-catenina).

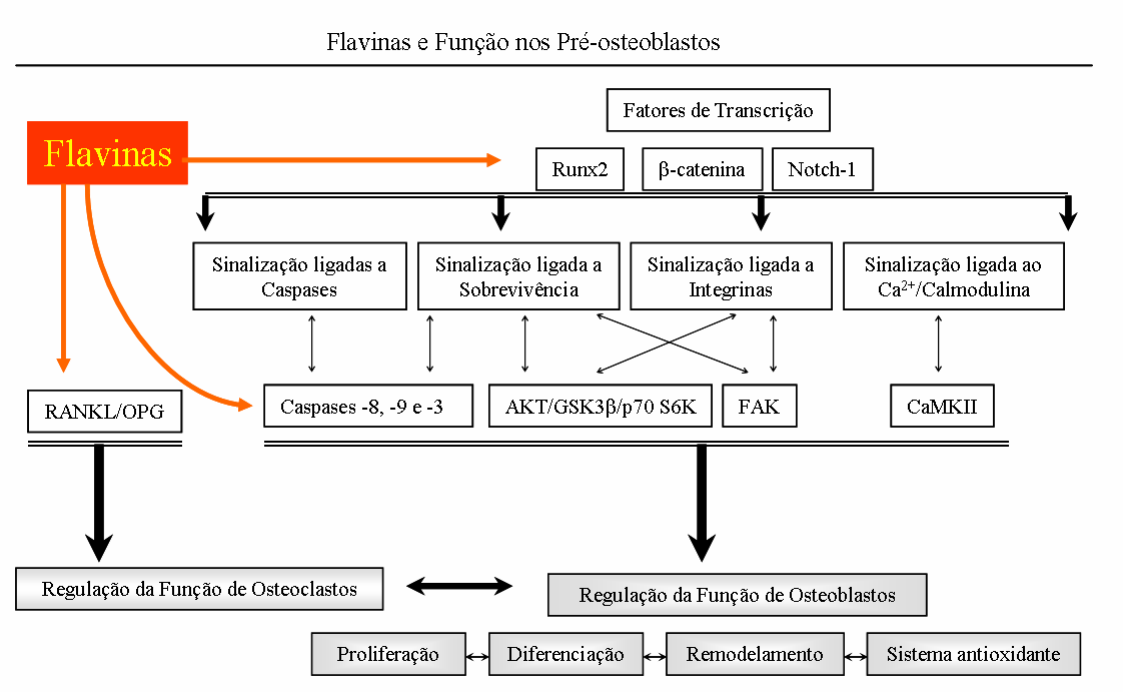

Figura 8. Mecanismo alvos de ação das flavinas na sinalização de osteoblastos.

Perspectivas

Perspectivas

A suplementação dietética com vitaminas do complexo B durante a senilidade, em especial a riboflavina, além de contribuir com a manutenção do equilíbrio sistêmico do estado oxidativo, poderia interagir diretamente com as células propiciando condições para atividade anabólica osteoblástica, o que contribuiria para a manutenção da saúde óssea e prevenção de doenças esqueléticas como a osteoporose. Os dados encorajam investigações futuras da associação da riboflavina e seu fotoprodutos com outras terapias para osteoporose, uma vez que i*n vitro* observamos a interação com indutores osteogênicos clássicos (ácido ascórbico e β-glicerofosfato) e subsequente efeitos aditivos na expressão de marcadores de diferenciação osteoblástica.

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Anexos

Anexos

Estágio no exterior

Estágio no exterior no Departamento de Imunologia-Biologia Celular do Centro Médico Universitário da Universidade de Groningen-Holanda de 01/03/2007 a 01/05/2007, sob a supervisão do Professor Maikel Petrus Peppelenbosch. O objetivo do estágio foi realizar a técnica do Pepchip que consta na metodologia do projeto Fapesp (2006/00430-1).

Trabalhos em Congressos

Chaves-Neto, A.H., Peppelenbosch, M.P., Ferreira, C.V. Biological evaluation of riboflavin and irradiated riboflavin in the MC3T3-E1 pre-osteoblastic cell line. VI International Congress of Pharmaceutical Sciences - Cifarp, Ribeirão Preto, Brasil, 02 a 05 de setembro de 2007.

Chaves-Neto, A.H., de Souza Queiroz, K.C., Peppelenbosch, M.P., Ferreira, C.V. Kinome profiling annotation of pre-osteoblast cells differentiation process by using arrays of quinase consensus substrates. 3rd FEBS Congress & 11th IUBMB Conference, Athens, Greece, 28 de junho a 03 de julho de 2008.

Colaborações

Participação na condição de Colaborador na elaboração da seguinte tese de doutorado "Influência da associação do plasma rico em plaquetas ao enxerto de osso autógeno no reparo ósseo de defeitos de tamanho crítico em calvária de coelhos", do doutorando Luiz Gustavo Nascimento de Melo, do Programa de Pós-Graduação em Odontologia – Área de Concentração Periodontia, da Faculdade de Odontologia de Araçatuba-UNESP (Orientadora: Professora Adjunto Maria José Hitomi Nagata). Os resultados deste trabalho foram submetidos para AAP-2007 (Annual Meeting American Academy Periodontology).

Participação na categoria de Professor Convidado da disciplina de Bioquímica dos cursos de odontologia integral e noturno da Faculdade de Odontologia de Araçatuba-UNESP em 2008, ministrando as seguintes aulas: Metabolismo do Fluoreto e Halitose.

Participação na categoria de Professor Convidado da disciplina de Fundamentos de Biologia Molecular (BB125) dos cursos do curso de Enfermagem da Universidade Estadual de Campinas em 2007 e 2008, ministrando a seguinte aula: Regulação da Expressão Gênica.

Desenvolvimento em paralelo trabalho baseado na análise dos efeitos da citotoxicidade dos fotoprodutos da riboflavina na linhagem de adenocarcinoma renal (786-0).