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Sinalização não-genômica do retinol mediada por espécies reativas

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Livros Grátis

Ubi dubium ibi libertas: Onde há dúvida, há liberdade

Provérbio latino

Agradecimentos

Índice

I.   Resumo e abstract

O retinol (vitamina A) exerce papéis fundamentais na regulação de processos celulares, tais como crescimento, divisão e apoptose. Os efeitos do retinol em nível celular são classicamente atribuídos à ativação de receptores nucleares da família dos receptores esteróides, conhecidos como RAR e RXR. Esses receptores são ativados por diferentes isômeros do ácido retinóico, que é considerado o produto mais biologicamente ativo da metabolização do retinol. No entanto, trabalhos recentes vêm identificando que o retinol também exerce funções biológicas por mecanismos independentes da transcrição gênica pela ativação desses receptores. Esta ação não clássica vem sendo chamada como ação não-genômica da vitamina A, e o mecanismo pelo qual isto acontece ainda é desconhecido. Neste trabalho, nós propomos que o mecanismo de ação não-genômica da vitamina A é mediado pela ativação redox dependente de rotas de sinalização citoplasmáticas, tais como ERK1/2, c-Src e PKC. Este efeito redox dependente está associado à modulação do cálcio extracelular e afeta a regulação do ciclo celular e ativação enzimática pós-transcricional.

Retinol (vitamin A) exerts fundamental roles in the regulation of cell processes such as growth, cell division and apoptosis. The effects of retinol at cellular level are classically ascribed to gene transcription mediated by nuclear receptors from the family of the steroid receptors, known as RAR and RXR. These receptors are activated by different isomeric forms of retinoic acid, the main metabolite of retinol. However, recent works have identified non-classical actions by retinol, involving mechanisms independent of the RAR/RXR-mediated gene transcription. These actions have been referred to as non-genomic actins of vitamin A, and the exact mechanism of these actions is still unknown. In the present work, we propose that the mechanism of non-genomic action by retinol involves the redox-dependent activation of cytoplasmic signaling pathways. This redox-dependent effect is associated to modulation of extracellular calcium and affects cell cycle regulation and post-transcriptional enzyme activation.

II. Siglas e abreviações

AP-1 – Proteína ativadora 1

ARAT - acil-CoA:retinol aciltransferase

Cdk – Cinase dependente de ciclina

CRALBP – Proteína celular ligadora de retinaldeído

CRABP – Proteína celular ligadora de ácido retinóico

cRaf-1 – Domínio regulatório rico em cisteína 1

CRBP – Proteina celular ligadora de retinol

CREB – Proteína ligadora de element responsivo à nucleotide cíclico

CYP26 – Citocromos P450 da família 26

DCFH - Diclorohidrofluoresceína

DR1, DR2, etc. – Repetição direta 1, 2, etc.

DTT - ditiotreitol

ERK1/2 – Cinases reguladas por sinal extracelular 1 e 2

JNK – *Jun* cinase

LRAT - lecitina:retinol aciltransferase,

LXR – Receptor hepático X

NAC – N-Acetil-cisteína

NF-κB – Fator nuclear *kappa* B

p38MAPK, ou p38 – Proteína cinase ativada por mitógenos p38

PI3K – Fosfatidil-inositol-3 cinase

PKA – Proteína cinase A

PKC – Proteína cinase C

PPAR – Receptor ativador de proliferação de peroxissomos

RA – ácido retinóico

RABP – Proteína ligadora de ácido retinóico

RALDH – retinal desidrogenase

RAR – Receptor de ácido retinóico

RARE – Elemento responsivo a ácido retinóico

RBP – Proteína ligadora de retinol

RDH – Retinol desidrogenase

REH – Retinol éster desidrogenase

RSK – Cinase ribossomal S6

RXR – Receptor retinóide X

TH – Tirosina Hidroxilase

III. Introdução

Vitamina A e os retinóides

A vitamina A (retinol) e seus análogos, conhecidos coletivamente como retinóides, exercem um papel essencial em processos de crescimento e diferenciação (Gudas et al., 1994). Por esta razão, os retinóides são reconhecidos reguladores de funções associadas à divisão celular e diferenciação, tais como reprodução, desenvolvimento embrionário e crescimento. Além disso, essas moléculas também estão envolvidas na manutenção de processos fisiológicos tais como a visão e funções motoras.

O termo geral "retinóides" compreende o retinol, considerada a "molécula-mãe" da família da vitamina A, bem como o ácido retinóico, que é formado intracelularmente através do metabolismo oxidativo do retinol, além de outros metabólitos naturais, como o retinaldeído (fig.1). Além disso, vários análogos sintéticos com similaridade estrutural ou funcional ao retinol também são reconhecidos como pertencentes à grande família dos retinóides. Os retinóides naturais são compostos isoprenóides de 20 carbonos com um anel beta-ionilideno, uma cadeia lateral de carbonos contendo ligações duplas que possibilitam variadas configurações isoméricas, e um grupo funcional terminal em um dos três estados de oxidação (Bollag, 1983).

Figura 1 – Retinóides. O retinol é gerado a partir do beta-caroteno via retinaldeído (retinal). Ambos podem gerar ácido retinóico, que por sua vez origina outros isômeros, através da ação das enzimas indicadas pelas setas.

A síntese *de novo* de retinol é restrita às plantas e a alguns microorganismos (Goodwin, 1963). Animais obtêm vitamina A da dieta, sob a forma de pró-vitamina A, ou como vitamina A pré-formada. Exemplos de pró-vitamina A são alguns compostos carotenóides, que são encontrados em diversos vegetais. O beta-caroteno, por exemplo, é convertido em retinol através de dois passos enzimáticos, na mucosa intestinal (Fig. 1a). Por outro lado, a principal forma de vitamina A pré-formada encontrada na dieta é o retinol esterificado a ácidos graxos de cadeia longa (chamados genericamente de ésteres de retinol), obtido através de alimentos de origem animal (principalmente fígado, leite e derivados – Olson, 2001), além de alimentos processados industrialmente e em suplementos vitamínicos. ?steres de

retinol são hidrolisados no lúmen intestinal por enzimas pancreáticas e da mucosa intestinal. Após esses processamentos enzimáticos da vitamina A pré-formada e da pró-vitamina A no lúmen intestinal, o retinol livre é absorvido pelas células mucosas e re-esterificado a ácidos graxos, geralmente saturados, de cadeia longa, no citoplasma das mesmas (através da ação da enzima lecitina:retinol aciltransferase, LRAT – MacDonald & Ong, 1988). Os ésteres de retinol resultantes desse processo são incorporados, com outros ésteres de lipídios neutros (por exemplo, triacilglicerídeos e ésteres de colesterol), nos quilomícrons, e absorvidos através do sistema linfático (Harrison & Mussain 2001).

Durante o processamento dos quilomícrons pelos tecidos extra-hepáticos, algumas células absorvem ésteres de retinol liberados pela ação da lipase lipoprotéica (Miano & Berk, 2000). Os quilomícrons remanescentes são, em seguida, captados pelo fígado, que é o principal órgão armazenador de vitamina A sob condições normais (Yost, Harrison & Ross, 1988). Neste órgão, o retinol é captado primeiramente pelos hepatócitos, onde uma hidrolase de ésteres de retinol (REH) hidrolisa esses compostos, gerando retinol livre. Este é complexado a proteínas citoplasmáticas ligadoras de retinol (*cellular retinol binding proteins*, ou CRBPs – Thompson & Gal, 2003).

Após esse processo de captação de retinol pelo hepatócito, o retinol em excesso é transportado por difusão para as células hepáticas estreladas, onde é re-esterificado pela ação da acil-CoA:retinol aciltransferase (ARAT) e pela LRAT, sendo

armazenado em gotas lipídicas citoplasmáticas, juntamente com outros lipídios neutros. Em condições normais, 80% do retinol hepático em um indivíduo normal é encontrado nas células estreladas, sendo o restante encontrado nos hepatócitos. O retinol hepático é mobilizado através da ação de uma REH nas células estreladas hepáticas, que hidrolisam os ésteres de retinol e os liberam para serem complexados a proteínas ligadoras de retinol plasmáticas (*retinol binding proteins*, RBPs), nos hepatócitos, para secreção na circulação (Soprano & Blaner 1994).

O transporte de retinol para os tecidos extra-hepáticos é realizado principalmente pelas RBPs. Dois modelos de captação celular de retinol foram propostos; o modelo da difusão propõe que o passo-limitante na captação de retinol é a lenta dissociação do *holo*-complexo retinol-RBP no ambiente extracelular (Hussain et al., 2001), enquanto que o modelo da captação mediada por receptor sugere que este é um processo específico, mediado pela interação da RBP com um receptor de membrana (Ross, 1993). Uma vez dentro da célula, o retinol complexa-se novamente com proteínas ligadoras de retinol (CRBPs)

O retinol citoplasmático tem diversos destinos metabólicos, que variam principalmente de acordo com a função celular. Nos hepatócitos, diversos tipos de metabólitos diretos do retinol são formados através da ação de enzimas do complexo citocromo P450 (Noy, 2000). Alternativamente, o retinol pode ser oxidado a 11-*cis*-retinal, composto de importância central no ciclo visual, pela retinol-desidrogenase (RDH). O 11-*cis*-retinal pode permanecer no citoplasma complexado a proteínas de

função homóloga às CRBPs (chamadas *cellular retinaldehyde binding proteins,* CRALBPs) ou ser oxidado a ácido retinóico pela retinal desidrogenase (RALDH). O ácido retinóico citoplasmático também se encontra associado a proteínas ligadoras específicas (*cellular retinoic acid binding proteins,* CRABPs), sendo que formas plasmáticas dessa proteína também já foram identificadas (Ross, 1993). A degradação enzimática do ácido retinóico é catalisada por enzimas da família 26 do grupo das citocromos P450 (denominadas CYP26). Além disso, o ácido retinóico, que é normalmente encontrado na forma "todo-*trans*", pode ser isomerizado em formas *cis*, como o ácido retinóico 9-*cis*, e o significado fisiológico exato desta transformação ainda é bastante debatido.

Mecanismos de Ação

Ação genômica

Sempre houve um consenso geral de que o mecanismo clássico de ação da vitamina A deveria envolver funções nucleares, controlando processos relacionados à regulação da expressão gênica, tais como diferenciação e crescimento. Esse mecanismo só foi elucidado a partir de 1987, com a clonagem dos receptores nucleares retinóides, e a subseqüente identificação de elementos responsivos (A/G)G(G/T)TCA-(n)-(A/G)G(G/T)TCA contendo alta afinidade por esses receptores na região promotora de certos genes (Chambon, 1994). Esses receptores, pertencentes à grande família dos receptores nucleares de hormônios

esteróides/tireóides/vitamina D, são sub classificados em dois grandes grupos: RAR (*retinoic acid receptors*) e RXR (*retinoid X receptors*), que funcionam como hetero-dímeros RAR-RXR (Kastner et al., 1997). Cada grupo consiste de três isoformas (α, β e γ) que são codificadas por diferentes genes (Chambon, 1994). Os RARs são ativados por ácido retinóico e seu isômero 9-*cis*, enquanto que os RXRs, inicialmente considerados receptores órfãos, foram observados serem ativados pelo ácido retinóico 9-*cis*, mas não pelo ácido retinóico todo-*trans* (Ross, 1993).

O elemento responsivo à acido retinóico (*retinoic acid response element*, RARE) mais freqüentemente encontrado na região promotora dos genes-alvo de RAR/RXR são aqueles contendo repetições diretas separadas por 5 pares de base (n=5; DR5), mas também são freqüentes os DR1 e DR2; além disso, homodímeros RXR-RXR também se ligam aos RARE DR1 (Mangelsdorf & Evans, 1995), sendo também capazes de formar heterodímeros com outros receptores nucleares, como PPAR (*peroxisome proliferator-activated receptor*) e LXR (*Liver X receptor* - Willy & Mangelsdorf, 1995). Como a modulação da expressão gênica pela vitamina A é mediada pela ativação de RARs e RXRs e subseqüente interação desses com os RAREs, é consenso na literatura que o ácido retinóico se constitui, entre os metabólitos naturais do retinol, no composto de maior importância biológica ativa, sendo inclusive citado em muitos trabalhos como a "forma ativa" da vitamina A, ou "vitamina A biologicamente ativa" (Zile, 2001). Esse mecanismo de ação via receptores retinóides RAR/RXR é referido atualmente, por essas razões, como mecanismo "genômico", ou "clássico", de ação biológica da vitamina A.

Ação não-genômica

Recentemente, tem-se observado que alguns dos efeitos causados pelo retinol *in vitro* e *in vivo* não são compatíveis com uma ação genômica, mediada por receptores retinóides. Esses efeitos são caracterizados principalmente por sua insensibilidade a inibidores de transcrição e síntese protéica, bem como velocidade de ação incompatível com o tempo necessário para se observarem alterações fisiológicas dependentes de ativação/repressão gênica. Estes fenômenos indicavam, assim, que a vitamina A também era capaz de exercer funções de maneira independente da ativação de receptores retinóides, ou pelo menos independente da modulação da transcrição gênica por esses receptores. Além disso, observou-se que outros agonistas de receptores nucleares de hormônios esteróides/tireóides/vitamina D também exercem ações similares (Pedram et al., 2002).

Têm-se sugerido que ações independentes da ativação de transcrição por retinóides têm uma importância fisiológica que foi negligenciada até recentemente. Já foi observado, por exemplo, que incubações com ácido retinóico induziam um bloqueio nos canais de potássio de linfócitos (Sidell & Schlichter, 1986). Na retina de vertebrados, o ácido retinóico também regula a transmissão de sinapses elétricas mediada por junções comunicantes (Zhang & McMahon, 2000), e em outros modelos

este fenômeno foi observado ser dependente da mobilização intracelular de cálcio (Liou et al., 2005).

Na presente década, diversos trabalhos vêm observando que, em diversos modelos de cultura de células, o tratamento com retinóides afeta drasticamente o estado de ativação da maquinaria de sinalização celular, em uma escala de tempo que varia entre segundos e minutos. Foi descrito que o ácido retinóico induz a ativação de ERK1/2 em células em 5 minutos de incubação (Cañon et al., 2004), levando à ativação do fator de transcrição CREB em células PC12. Além disso, a ativação de CREB por ácido retinóico foi observada também em células epiteliais de brônquios humanos, sendo mediada por uma rápida ativação de PKC, ERK e RSK (Aggarwal et al., 2006). Essas ações não-clássicas da vitamina A têm sido referidas na literatura como sendo o mecanismo de ação "extra-nuclear", ou "não-genômica", dos retinóides, e a natureza desse efeito ainda não foi esclarecida, havendo diferentes hipóteses correntes na literatura para explicar esses mecanismos de ação.

Mecanismos de ação não-genômica

Há um consenso na literatura de que a ação não-genômica da vitamina A, por definição, não envolveria transcrição gênica mediada por receptores retinóides (RAR-RXR). No entanto, isso não significa que esses receptores não estão envolvidos nesse fenômeno, como sugerem alguns autores. Inclusive, teoricamente, seria até possível que um dos efeitos, em longo prazo, da ativação de rotas de sinalização não-

genômica da vitamina A fosse a modulação da própria modulação da transcrição gênica mediada por esses receptores. Foi demonstrado, por exemplo, que certas rotas de sinalização podem alterar diretamente a atividade desses receptores, como é o caso das rotas da p38MAPK , cdk e JNK (Bastien & Rochette-Egly, 2003). No entanto, a sinalização não-genômica da vitamina A refere-se à modulação de quaisquer rotas de sinalização que resultem em fenômenos pós-tradução, tais como modulação sináptica e regulação enzimática, como também a modulação de rotas de sinalização envolvidas na modulação da expressão gênica por diversos fatores de transcrição, entre eles os próprios receptores retinóides.

Os poucos (e recentes) trabalhos com sinalização não-genômica da vitamina A indicam duas possibilidades para explicar o mecanismo desse tipo de sinalização, uma envolvendo a participação direta de receptores retinóides na ativação de rotas de sinalização citoplasmáticas, e outra excluindo essa participação. Todos estes trabalhos apresentam consistentes evidências experimentais demonstrando a participação ou não dos receptores retinóides na ação não-genômica da vitamina A, e é possível ainda que ambos os mecanismos não sejam excludentes.

Cañon et al. (2004), utilizando linhagens mutantes de PC12 que super-expressam receptores RARα e RARβ, sugeriram que esses receptores eram capazes de interagir diretamente e conseqüentemente ativar ERK1/2. No entanto, foi observado que tanto a ativação de ERK1/2, como também de PKC, ocorre também em células com receptores retinóides silenciados com RNA interferente, bem como na

presença de antagonistas de RAR/RXR (Aggarwal et al., 2006). Corroborando a hipótese de não-envolvimento de RAR/RXR na ativação de rotas de sinalização não-genômica, estudos cristalográficos mostraram a existência de um motivo de alta afinidade por ácido retinóico em PKC (Radominska-Pandya et al., 2000), e que o ácido retinóico efetivamente liga-se em dois sítios diferentes na região C2 da PKCα (Ochoa et al., 2003). Além disso, foi constatado que regiões ricas em cisteína de domínios regulatórios de cRaf-1 e das isoformas α, δ, μ e ζ de PKC servem como sítios de ligação de retinol, mas não de ácido retinóico, e que essa ligação influencia a ativação induzida por radicais livres nessas enzimas (Hoyos et al., 2000).

No entanto, neste ano, novos trabalhos renovaram a hipótese de participação dos receptores retinóides na ativação não-genômica de rotas de sinalização celulares. Masiá et al. (2007) demonstraram, utilizando técnicas de imunoprecipitação, que a ativação de PI3K por ácido retinóico em células SH-SY5Y é dependente da formação de um complexo estável da subunidade regulatória p85 e da subunidade catalítica p110 da PI3K com RARα. Além disso, também foi descrita a formação de complexos citoplasmáticos c-Src/RARγ na ativação da rota da CSK em linhagens LA-N-5 (Dey et al., 2007). Esses dados indicam a necessidade de mais estudos para esclarecer a natureza dos mecanismos não-genômicos de ação da vitamina A.

Retinol e espécies reativas: possível relação no mecanismo de sinalização não-genômica

Além das ações sinalizadoras discutidas acima, as propriedades redox ativas da vitamina A vêm despertando a atenção de pesquisadores desde meados da década de 80, quando se começou a especular que essas propriedades poderiam explicar grande parte dos seus efeitos biológicos (Diplock, 1991; Brown & Goodman, 1998). Inicialmente, sugeria-se que a vitamina A exercia um papel primariamente antioxidante, e que a diminuição de seu conteúdo no organismo poderia aumentar uma susceptibilidade geral a problemas relacionados ao desequilíbrio da produção de espécies reativas de oxigênio e estresse oxidativo (Jeandel et al., 1989). A partir de observações coletadas de estudos epidemiológicos, farmacológicos e bioquímicos, no entanto, chegou-se à conclusão de que a vitamina A também pode exercer um potencial papel pró-oxidante, aumentando inclusive a incidência de patologias relacionadas a mutações do DNA, possivelmente decorrentes do dano oxidativo observado em modelos *in vitro* (Murata & Kawanishi, 2000; Goodman et al., 1993; Omenn et al., 1994). A observação de que a vitamina A pode exercer tanto um papel pró-oxidante como antioxidante indica que o retinol pode ser classificado como uma molécula *redox-ativa*, ou seja, apresenta atividade redutora ou oxidante dependendo do micro-ambiente em que se encontra.

A indução de fenômenos dependentes da modulação de rotas de sinalização celular por retinol, já foi observada concomitantemente com a produção de espécies reativas de oxigênio e indução de estresse oxidativo. Em fibroblastos, tanto o retinol

como o retinal induzem apoptose dependente de estresse oxidativo (Gimeno et al., 2003). Nosso grupo constatou que, em células de Sertoli, o retinol aumenta a produção de superóxido mitocondrial, induzindo liberação de citocromo *c* da mitocôndria, e aumentando diversos parâmetros indicativos de estresse oxidativo, como lipoperoxidação, dano oxidativo em DNA, quantidade de proteínas carboniladas, e atividade de enzimas antioxidantes (Dal-Pizzol et al., 2001, Klamt et al., 2003). Além disso, a ocorrência de fenômenos relacionados a alterações do ciclo celular, como indução de apoptose e aumento de proliferação, também foram observadas. Esses dados, juntamente com dados disponíveis na literatura demonstrando a presença de domínios redox-sensíveis em diversas proteínas integrantes das rotas de sinalização celular - entre essas, proteínas que são ativadas de maneira não-genômica pela vitamina A, como as ERK1/2 e PKC (Aggarwal et al., 2006), inspiraram a hipótese de trabalho defendida nesta tese de doutorado.

IV. Objetivos

Justificativa e apresentação dos objetivos

Considerando que: **1)** o mecanismo de ação não-genômica da vitamina A não está definitivamente esclarecido, como exposto na Introdução; **2)** apesar de o potencial redox da vitamina A já ser reconhecido como um tendo um papel importante no seu mecanismo de ação biológica, como mencionado também acima, este potencial é desconsiderado, até o presente momento, por todos os trabalhos investigando a natureza do mecanismo de sinalização não-genômica da vitamina A; e que **3)** diversos fenômenos reconhecidamente regulados por rotas de sinalização citoplasmáticas são controlados de maneira redox-dependente em células incubadas com vitamina A, o **objetivo central desta tese foi o de determinar a importância das espécies reativas produzidas pelo tratamento com vitamina A na ativação de rotas não-genômicas de sinalização em diferentes modelos de cultura celular**, tendo como objetivos específicos:

a) Investigar a produção de espécies reativas em células incubadas com vitamina A (retinol) e com ácido retinóico, em tempo real;

b) Determinar a importância de espécies reativas na ativação de uma rota não-genômica de sinalização que resulte na modulação tardia da expressão gênica (modulação do ciclo celular em células de Sertoli);

c) Determinar a importância de espécies reativas na ativação de uma rota não-genômica de sinalização que resulte na modulação rápida de um fenômeno pós-transcricional (ativação da enzima Tirosina-Hidroxilase por fosforilação em células cromafins).

Parte 2 - Resultados

Capítulo I.

Evidence of increased intracellular reactive species formation by retinol, but not retinoic acid, in PC12 cells

Evidence of increased reactive species formation by retinol, but not retinoic acid, in PC12 cells

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Abstract

The biological effects of vitamin A (retinol) are generally ascribed to the activation of nuclear retinoid receptors by retinoic acid (RA), considered the most biologically active retinoid. However, it is not established whether the cytotoxic effects of vitamin A are due to retinoid receptors activation by RA. Vitamin A-related toxicity is associated with cellular redox modifications, often leading to severe oxidative damage, but the role of RA in this effect is also uncertain. We therefore studied the formation of intracellular reactive species induced by retinol and retinoic acid in PC12 cells, using an *in vitro* dichlorofluorescein (DCFH) fluorescence real-time assay. We observed that retinol, but not retinoic acid, induced a steady increase in DCF-based fluorescence over 60 min of incubation, and this increase was reversed by antioxidant (N-acetyl-cysteine and α-tocopherol) pre-treatment. This effect was also inhibited by the iron chelator 1,10-phenantroline and the impermeable calcium chelator EGTA. These results suggest that vitamin A-associated cytotoxicity is probably related to an oxidant mechanism dependent on iron and calcium, and the formation of intracellular reactive species is related to retinol, but not to RA.
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Keywords: Retinol; Retinoic acid; Intracellular reactive species; DCFH; PC12

1. Introduction

Vitamin A (retinol) is an essential factor for regulation of cell cycle and differentiation during early stages of development. Retinol can be converted to retinoic acid (RA), which is the main activator of nuclear steroid receptors known as retinoid receptors. These receptors are subdivided into retinoic acid receptors (RAR) and retinoid X receptors (RXR) and it is generally accepted that most of the biological actions of retinol are mediated by the modulation of these receptors, after the conversion of retinol into RA. However, other studies have shown that retinol and RA can exert some of their effects by triggering cellular events not related to retinoid receptor activation; these events have been referred to as non-genomic or extra-nuclear effects of retinoids (Liao et al., 2004; Elliot, 2005).

Retinoids have redox-related properties and influence the oxidant status of the cell (Murata and Kawanishi, 2000; Klamt et al., 2003). Many authors suggested that retinol and related molecules, such as beta-carotene, act in biological systems as antioxidants and thus could be potential clinical agents in antioxidant therapies for treatment and prevention of malignant and neurodegenerative diseases (Okuno et al., 2004). However, clinical trials have observed that retinoids can also be deleterious and are associated with activation of proto-oncogenes, leading to an increased incidence of neoplasias (Omenn, 2007). The existence of these apparently conflicting data implies that more studies are necessary to address this issue. This is especially so when it is considered that retinoid-based therapies present potential treatments for a range of diseases related to cell cycle disruption/cell death and

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increased reactive oxygen species (ROS) formation, including skin cancer, lung cancer, Parkinson's disease and Alzheimer disease (Njar et al., 2006; Greenwald et al., 2006; Ono and Yamada, 2007).

Our group has been studying the effects of retinol on oxidative stress-related parameters, and we observed that retinol induced several cytotoxic parameters. We reported that retinol increased lipoperoxidation, DNA oxidative damage, mitochondrial superoxide production and led to mitochondrial membrane swelling and cytochrome c release, causing cell death (Dal-Pizzol et al., 2000; Dal-Pizzol et al., 2001; Klamt et al., 2005). Furthermore, retinol activated ERK1/2 and CREB, causing proliferative focus formation, and these effects were reversed by antioxidant treatment (Gelain et al., 2006). Interestingly, these results were always observed with the administration of 7 μM retinol, while concentrations up to 5 μM – which is considered to be within the physiological range for cells (Ross, 1993) – were never observed to induce deleterious effects. This suggested that slight variations in the concentrations of retinol may trigger changes in the cellular redox state. These results indicate that retinol can also induce toxic effects, not compatible with the genomic action of retinoid receptors, and these effects are, at least in part, mediated by intracellular reactive oxygen species.

It is not known whether the pro-oxidant properties observed with retinol are due to its conversion to RA. The fact that this conversion is by many different non-specific cellular dehydrogenases (Lidén et al., 2003) also made difficult an approach using enzymatic inhibitors.

In the present study, we report that retinol, but not RA, increases intracellular reactive species formation in cultured PC12 cells, using a real-time DCFH-DA based assay for intact, living cells (Wang and Joseph, 1999). This is the first time that increased production of intracellular reactive species by retinol is demonstrated in PC12 cells, and also that RA is shown not to increase the formation of these species by the same approach. Also, we observed that EGTA (calcium chelator) and 1,10-phenantroline (iron chelator) inhibited the effect of retinol, suggesting that the presence of calcium and iron is essential for retinol-induced toxicity.

2. Material and methods

2.1. Chemicals

EGTA, *N*-acetyl-cysteine (NAC), all-*trans* retinol alcohol, all-*trans* retinoic acid (RA), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA) and 1,10-phenantroline were from Sigma Chemical Co. (St Louis, MO, USA). Retinol and RA were dissolved in ethanol. Concentrated stocks were prepared immediately before experiments by diluting retinol or RA into ethanol and determining final stock concentration by UV absorption; solutions were kept protected from light and temperature during all procedures.

Appropriate solvent controls were performed for each condition. Retinol and RA treatments were initiated by adding concentrated solutions to reach final concentrations in the well. The final ethanol concentration did not exceed 0.2% in any experiment. Tissue culture reagents were from Sigma Chemical Co. and were of analytical or tissue culture grade.

2.2. Cell culture

PC12 cells were originally obtained from the ATCC (VA, USA) and were kindly donated by Dr. Peter R Dunkley (University of Newcastle, Australia) and Dr. Alicia Kolwaltolwski (Universidade de Sao Paulo, Brazil). Cells were plated onto plastic culture plates and maintained under a 5% CO_2-containing atmosphere at 37 °C in the presence of complete medium (Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 5.5 mM glucose, 10% fetal bovine serum, 5% fetal horse serum, 15 mM HEPES, pH 7.4, 100 mg/mL penicillin G, 100 mg/mL streptomycin, 50 mg/mL gentamicin and 5 mg/mL Mycostatin) for 24–48 h, or until cells reached 70–80% confluence. Preliminary MTT viability assay indicated that cell viability was not affected during incubations.

2.3. DCFH-DA assay

Intracellular ROS production was determined by the DCFH-DA-based real-time assay using intact living cells, as described by Wang and Joseph (1999). Briefly, PC12 cells were plated onto 96-well plates and incubated for 1 h with DCFH-DA 100 μM (stock solution in DMSO, 10 mM) in 1% FBS culture medium at 5% CO_2 and 37 °C. Then cells were washed and treatments were carried out. During treatment, changes in the fluorescence by the oxidation of DCFH into the fluorogen DCF were monitored in a microplate fluorescence reader (F2000, Hitachi Ltd., Tokyo, Japan) for 1 h at 37 °C. H_2O_2 1 mM was used as positive control for ROS production. Excitation filter was set at 485 ± 10 nm and the emission filter was set at 530 ± 12.5 nm. Data were recorded every 30 s and plotted in Excel software.

2.4. MTT assay

Following retinol treatment, PC12 cells viability was assessed by the MTT assay. This method is based on the ability of viable cells to reduce MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.2 mg/mL. The cells were left for 45 min at 37 C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm (reference). H_2O_2 1 mM was used as positive control.

3. Results

Real-time DCFH-DA assay showed that retinol induced a concentration-dependent steady increase in intracellular reactive species in PC12 cells over 1 h of incubation (Fig. 1A). While concentrations up to 2.5 μM retinol did not have any effect, we observed increased formation of intracellular reactive species with 5 μM and 10 μM. The rate of increase in DCF fluorescence shown in Fig. 1B was calculated over this first hour of incubation. This range of concentrations was chosen based on physiological levels reported for cells (Ross, 1993). A similar experiment using RA (0.1–10 μM) was performed. However, RA failed to produce any effect upon PC12 cells over 1 h of incubation (not shown). Fig. 1C and D shows DCF-based fluorescence in cells treated with retinoic acid (10 μM) in comparison to cells incubated with retinol (10 μM) and H_2O_2 (1 mM, used as a positive control for DCF fluorescence). Vehicle (ethanol 0.2%) had no effect on this assay.

Next, we verified the effect of different antioxidant pre-treatments (30 min) on the retinol-induced increase in DCF fluorescence. Both N-acetyl-cysteine (NAC) 1 mM and α-tocopherol 0.1 mM inhibited the effect of retinol (Fig. 2A and B), indicating that antioxidant pre-treatment is able to inhibit the pro-oxidant effect of retinol. This result agrees with our previous observations, where antioxidants inhibited DNA damage and apoptosis caused by retinol treatment (Dal-Pizzol et al., 2001).

We previously observed that DNA damage induced by retinol is dependent on iron uptake, and that iron chelators inhibited DNA damage by retinol treatment (Dal-Pizzol et al., 2001). Recent observations also suggested that calcium might be involved in the pro-oxidant effects of retinol (Klamt et al., 2005). In this regard, we verified the effect of pre-incubation with the iron chelator 1,10-phenantroline (0.1 mM) or the calcium chelator EGTA (4 mM) on the effect of retinol. Both treatments inhibited retinol-induced DCF fluorescence (Fig. 2C and D). Combination of both treatments further decreased intracellular reactive species production. This result suggests that iron and calcium were involved in the production of cellular free radicals induced by retinol. Viability measurements by the MTT assay
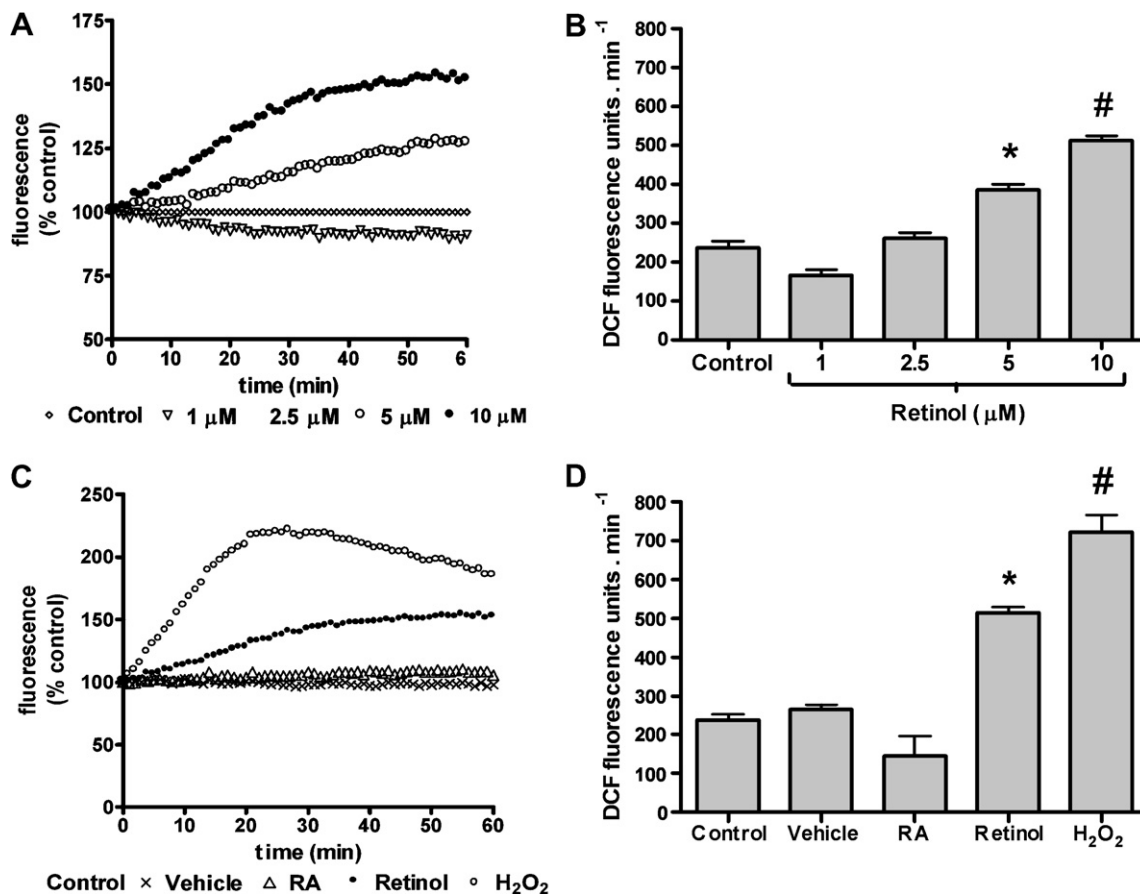

Fig. 1. Kinetics of intracellular reactive species production in PC12 cells evaluated by the DCFH-DA real-time fluorescence assay. (A) PC12 cells were incubated with increasing concentrations of retinol (up to 10 μM) for 60 min. (B) The rate of DCF-based fluorescence production over this period was plotted and analysed. (C) Kinetics and (D) rate of DCF-based fluorescence production were compared between PC12 cells treated with only ethanol 0.2% (vehicle), retinoic acid 10 μM (RA), retinol 10 μM (retinol) and H_2O_2 1 mM. Data are representative of 3 different experiments (A and C, each line is the mean of 16 different wells) or expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software (B and D). $^*p < 0.05$, $^\#p < 0.01$.
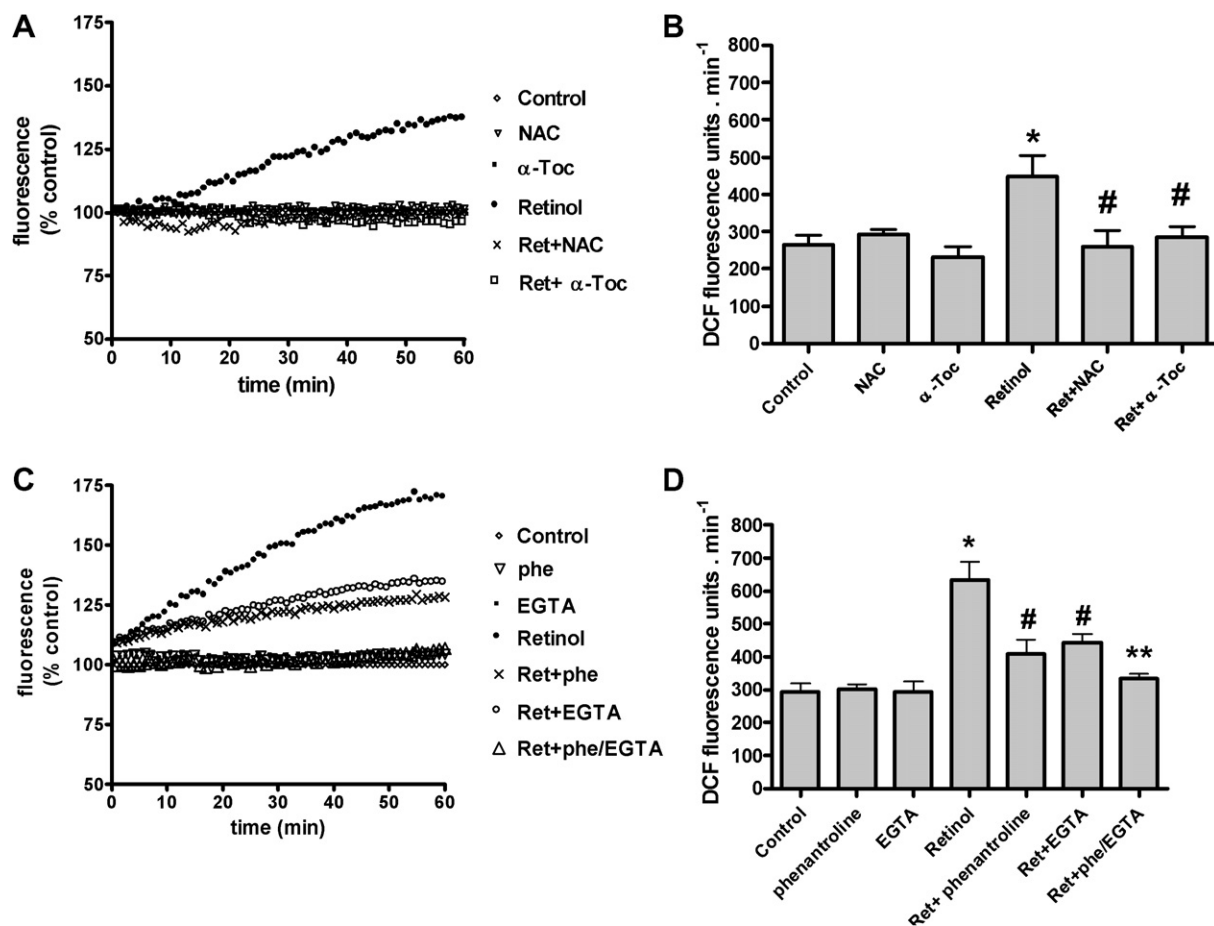
Fig. 2. Effect of antioxidants and iron and calcium chelators on retinol-induced intracellular reactive species formation. The effect of 30 min pre-incubation with (A) N-Acetyl-cysteine 1 mM (NAC) and α-tocopherol 0.1 mM, or (C) the iron chelator 1,10-phenantroline 0.1 mM (phe) and the calcium chelator EGTA 4 mM on DCF-based fluorescence over 60 min of retinol 10 μM incubation is shown. The rate of DCF-based fluorescence increase during this time course was analysed (B and D). Data are representative of 3 different experiments (A and C, each line is the mean of 16 different wells) or expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software (B and D). $^*p < 0.01$, $^#p < 0.05$, $^{**}p < 0.001$.

indicated no alteration on cell viability by retinol (10 μM) or RA (1 μM) after 60 min of incubation; however, after 24 h, a decrease in cellular viability was observed in retinol-treated cells (Fig. 3).
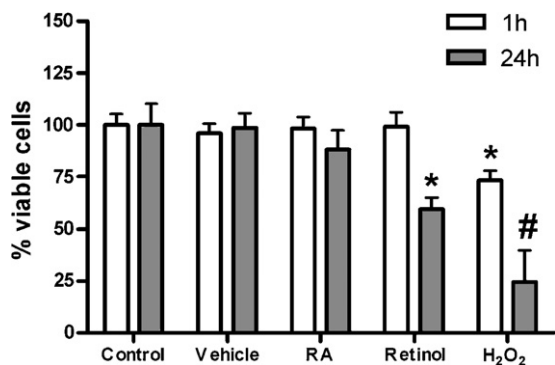

Fig. 3. MTT assay. Cellular viability was assessed by MTT assay after 1 h or 24 h of ethanol 0.2% (vehicle), retinoic acid 10 μM (RA), retinol 10 μM (retinol) and H_2O_2 1 mM incubation. Data are expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software. $^*p < 0.05$, $^#p < 0.01$.

4. Discussion

The ability of retinol and related molecules to act as antioxidant or protective agents in biological systems has been strongly supported in recent years. Several studies have reported a correlation between low serum levels of retinoids, or carotenoids, and the incidence of pathologies that are related in part to increased ROS production, such as neurodegenerative diseases and neoplasias (Jimenez-Jimenez et al., 1993; Greenwald et al., 2006). The mechanisms underlying these apparent protective properties are still not well understood. Clinical trials were undertaken aimed at establishing the efficiency of retinoid/carotenoid-based antioxidant therapies against lung cancer (the ATBC and the CARET trials); however these studies had to be discontinued due to the increased mortality related to lung cancer and cardiovascular disease incidence caused by the experimental treatments (Omenn et al., 1994). More recent studies observed that retinol or related molecules could exert pro-oxidant and cytotoxic effects, both *in vitro* and *in vivo* (Murata and Kawanishi, 2000; Pennis-

ton and Tanumihardjo, 2006). The mechanisms of these effects are also poorly understood.

This is the first time that reactive species production is demonstrated to be increased by retinol incubation in intact living cells. DCFH is commonly used as a probe to evaluate intracellular ROS formation; it was first described as a hydrogen peroxide-specific probe, but later it was shown to be sensitive to other reactive species as well, including hydroxyl and peroxyl radicals (LeBel et al., 1992). Nevertheless, it is demonstrated to be an excellent index for quantification of cellular oxidative stress, since it is oxidized intracellularly and the emitted fluorescence was demonstrated to be directly proportional to the production of intracellular reactive species. This is an advantage in relation to other methods that evaluate oxidative stress indirectly by the quantification of damaged biomolecules (Wang and Joseph, 1999). The assay used in this study is an adaptation to the original technique, which allows it to work with intact living cells, and to assess the production of ROS, besides other reactive species, in real time (Wang and Joseph, 1999). In this regard, the data presented here strongly indicate suggest that retinol is able to induce oxidative stress in cell systems by increasing the production of free radicals in a concentration-dependent manner.

It has been reported by different groups that other retinoids than vitamin A itself have pro-oxidant and/or toxic properties. Beta-carotene was observed to stimulate human pulmonary adenocarcinoma cells by activation of PKA and ERK1/2 pathways (Al-Wadei et al., 2006), to induce haem oxygenase-1 in HFP-1 cells (Obermuller-Jevic et al., 1999) and to increase ROS production and decrease cellular oxidised glutathione content in cancer cell lines (Palozza et al., 2003). Murata and Kawanishi (2000) observed the occurrence of DNA oxidative damage *in vitro* caused by retinol and retinal. In this study we observed that retinol had a very clear effect increasing ROS production in a time- and concentration-dependent manner. In contrast, a wide range of concentrations of RA failed to produce any effect. However, RA did not seem to exert antioxidant effects in these cells, since it has not decreased basal reactive species production; also, RA co-incubation with retinol had not affected retinol-induced intracellular reactive species production (data not shown).

It has long been postulated that RA is the most active metabolite of retinol in biological systems; many authors refer to RA simply as the "metabolically active vitamin A" (Zile, 2001). This concept is related to the fact that, among the metabolites of retinol, only all-*trans* RA and 9-*cis* RA are able to activate gene transcription through the activation of the RAR and RXR retinoid receptors (Chambon, 1994). However, it has becoming increasingly clear that vitamin A-related compounds, including RA, exhibit biological activities other than RAR/RXR-mediated gene transcription (Ochoa et al., 2003; Aggarwal et al., 2006). Despite the observations that retinol administration to cells and vitamin A supplementation protocols

may lead to increased oxidative damage or toxicity (Dal-Pizzol et al., 2001; Omenn et al., 1994), the question about whether these effects are caused by the action of retinol itself or by the RA generated after retinol administration/intake has never been explored. Here, we have not observed any similarities between the effect of retinol and RA on cellular reactive species production by the DCFH assay. This suggests that vitamin A may exert different effects on cell systems by the action of different derivatives from retinol, including retinol itself. These actions may well involve both genomic and non-genomic mechanisms.

We observed that retinol-dependent ROS production was inhibited by iron and calcium chelators. We had previously noted that iron was involved in the oxidant-mediated cytotoxicity by retinol (Dal-Pizzol et al., 2000), but this is the first time we observed the involvement of calcium on intracellular reactive species production by retinol. Since EGTA is not permeable to cell membranes, this effect is probably dependent on extracellular calcium. The involvement of iron in ROS production is generally associated to the transition metal-catalyzed Fenton reaction, which accounts for the production of highly reactive hydroxyl radicals from previously formed hydrogen peroxide, leading to severe oxidative damage (Terman and Brunk, 2006). However, there are no reports linking extracellular calcium to increased ROS formation in cells. DCFH reacts intracellularly with both hydroxyl radicals and hydrogen peroxide, thus the inhibition of iron-mediated radicals production by the iron chelator 1,10-phenantroline is probably diminishing the DCF fluorescence associated only to hydroxyl radicals, but not to hydrogen peroxide. As hydrogen peroxide production is also probably increased by retinol, since this compound is necessary for iron-mediated hydroxyl formation, the remaining DCF fluorescence in cells pre-treated with the iron chelator is most likely due to hydrogen peroxide formed by a mechanism dependent on extracellular calcium, since combined EGTA and 1,10-phenantroline further inhibit ROS production by retinol.

In conclusion, this is the first work demonstrating intracellular reactive species production by retinol using intact, living cells, by a real-time fluorescence-based assay, and the first time that retinol-induced ROS formation was observed to be dependent on both iron and calcium. We did not observe any pro-oxidant effect by RA, indicating that most pro-oxidant and/or redox activities associated to vitamin A are not related to the *in vivo* production of RA from retinol. This may also explain the decreased cellular viability observed after 24 h of retinol incubation, which was not observed with RA.

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Capítulo II.

Retinol induces the ERK1/2-dependent phosphorylation of CREB through a pathway involving the generation of reactive oxygen species in cultured Sertoli cells

Retinol induces the ERK1/2-dependent phosphorylation of CREB through a pathway involving the generation of reactive oxygen species in cultured Sertoli cells

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Abstract

The ability to regulate cell cycle progression and apoptosis through the activation of nuclear receptors and gene transcription has been generally accepted as a potential chemopreventive and therapeutic property of retinoids. However, recent studies suggest that retinol and related compounds can exert rapid and non-genomic effects, which may increase the production of reactive oxygen species (ROS) and lead to cell cycle disruption and malignant transformation. In this work, we report that, in Sertoli cells, retinol (7 μM) induces the Src-dependent activation of ERK1/2 MAPK and the ERK1/2-mediated phosphorylation of the transcription factor CREB. We found that these retinol-induced effects were completely blocked by the antioxidant Trolox 100 μM (a hydrophilic analogue of alpha-tocopherol), the hydroxyl radical scavenger mannitol (1 mM) and the addition of native superoxide dismutase (200 U/ml), and also that retinol increased the production of ROS and several other parameters indicative of oxidative stress during the same incubation periods in which ERK1/2 and CREB were phosphorylated. The activation of the ERK1/2–CREB pathway appears to be involved in the onset of some of the malignant effects caused by retinol in Sertoli cells since inhibition of ERK1/2 activation blocked the retinol-induced cell transformation and proliferation.
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Keywords: Retinol; ERK1/2; CREB; Src; Sertoli cells; Oxidative stress

1. Introduction

It is known that vitamin A (retinol) plays an essential role during embryogenesis and in the maintenance of functions such as vision, growth, differentiation and reproduction [1]. Although retinol and its derivatives, the retinoids, have been considered as potential preventive and therapeutic agents for malignant diseases [2–4], mainly because of their ability to regulate cell cycle progression, differentiation and apoptosis in transformed/malignant cell lines [5,6], the effects of retinol supplementation are still incompletely characterized. While some authors suggest the existence of a positive correlation between retinol consumption and cancer prevention [7–10], others indicate a link between retinoids and the activation of a variety of proto-oncogenes which can contribute to cellular transformation [11–13].

Indeed, several evidences suggest that the retinoids have pro-oxidant properties, which might lead to cellular oxidative damage and carcinogenesis [14,15]. For example, we observed that incubation of Sertoli cells with retinol (7 μM, 24 h) causes genotoxicity, recombinogenicity and cellular preneoplastic transformation; besides, it increases mitogenic signaling and the chromatin sensitivity to DNAse I [16–19]. We also observed

that retinol leads to an increase in iron uptake by the cell, which enhanced DNA oxidative damage and cell lipoperoxidation, probably by generation of the hydroxyl ($\cdot$OH) radical through Fenton chemistry, since both iron chelators and $\cdot$OH scavengers inhibited this effect [20–22]. Recently, we demonstrated that retinol treatment, by increasing superoxide production, modifies mitochondrial architecture and physiology, leading to membrane swelling and cytochrome *c* release to the cytoplasm [23]. These previous observations strongly indicate that retinol has a cytotoxic and/or pro-neoplasic role which is at least partially mediated by an increment in reactive oxygen species (ROS) production.

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the superfamily of mitogen-activated protein kinases (MAPK). These kinases are important regulatory proteins through which extracellular signals are translated into intracellular events [24,25]. After being activated by phosphorylation at Thr and Tyr residues, ERK1/2 phosphorylate cytoplasmic proteins, such as signaling enzymes, other kinases, cytoskeletal proteins and, importantly, translocate to the cellular nucleus where they activate, directly or indirectly, several transcription factors, including the cAMP-responsive element binding protein (CREB) [26], thereby regulating the expression of specific genes [27]. ERK1/2 appear to play a major role in cell proliferation and differentiation [28–30] as well as in oncogenetic transformation [31–33] and it has been reported that free radicals are able to up-regulate ERK1/2 signaling [34–36].

Since we had previously demonstrated that retinol increases mitogenic signaling through the production of ROS, thus leading to proliferation focus formation in Sertoli cells [22], we decided to analyze whether retinol can modulate the activation state of ERK1/2–CREB signaling pathway. We found that incubation of Sertoli cells with retinol (7 μM) induces a rapid and transient increase in ERK1/2 and CREB phosphorylation, which is dependent on upstream Src-tyrosine kinase and MAPK/ERK kinases 1/2 (MEK1/2) activities. Moreover, co-treatment of retinol with the antioxidant agents Trolox (a hydrophilic analogue of vitamin E), mannitol (an $\cdot$OH radical scavenger) and with native superoxide dismutase enzyme (SOD) inhibited the effect of retinol on ERK1/2–CREB pathway. Also, we found that inhibition of the ERK1/2 phosphorylation pathway hindered both retinol-induced proliferative focus formation and the retinol-promoted phosphorylation of CREB. Our data suggest that retinol might be able to regulate cell cycle progression through a mechanism involving the redox-sensitive activation of the ERK1/2 pathway.

2. Materials and methods

2.1. Materials and animals

Anti-ERK, and anti-phospho-ERK1/2 (Thr202/Tyr204) were obtained from Cell Signaling Technology (Beverley, MA, USA), anti-CREB, anti-phospho-CREB (Ser133), anti-MEK1/2, and anti-phospho-MEK1/2 (Ser217/Ser221) were from New England Biolabs (USA), anti-phospho-Tyr417-Src from Upstate Biotechnology (Lake Placid, NY, USA) and anti-tubulin was from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase-coupled anti-IgG

antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The West Pico chemiluminescent kit was obtained from Pierce (Rockford, IL, USA). Other drugs, kits and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Pregnant Wistar rats were housed individually in plexiglass cages. Litters were restricted to eight pups each. The animals were maintained on a 12-h light/dark cycle at a constant temperature of 23 °C, with free access to Purina lab chow and water. Male immature rats (15 days old) were killed by cervical dislocation.

2.2. Isolation and culture of Sertoli cells

Sertoli cells were isolated as previously described [37]. Briefly, testes of 15-day-old rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 34 °C, and centrifuged at 750×g for 5 min. The pellet was mixed with soybean trypsin inhibitor, then centrifuged and incubated with collagenase and hyaluronidase for 30 min at 34 °C. After incubation, this fraction was centrifuged (10 min at 40×g). The pellet was taken to isolate Sertoli cells and the supernatant was discarded. After counting, Sertoli cells were plated in 6×dishes multiwell plates (3×10^5 cells/cm^2) in Medium 199 pH 7.4 1% FBS, and maintained in a humidified 5% CO$_2$ atmosphere at 34 °C for 24 h to attach. The medium was then changed to serum-free medium and cells were taken for assay after 24 h of culture. Sertoli cell cultures were estimated to be 90–95% pure, as assessed by the alkaline phosphatase assay. Assays consisted in treating cells for different times with varying concentrations of retinol with or without the standard antioxidant Trolox® (a hydrophilic analogue of vitamin E, 100 μM), the $\cdot$OH radical scavenger mannitol (1 mM), the thiol antioxidants dithiothreitol (DTT; 1 mM) and *N*-acetyl-cysteine (NAC, 1 mM), the iron chelator 1,10-phenantroline (100 μM), and with native purified superoxide dismutase (SOD, EC 1.12.1.1, 200 U/ml) – denatured SOD (dSOD, boiled for 5 min to destroy enzyme activity) was used at the same concentration as a control for SOD treatment. The ERK1/2-kinase (MEK1/2) inhibitor PD98059 10 μM and the Src-tyrosine kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) 10 μM, when utilized, were always co-administered with retinol.

2.3. Immunoblot

To perform immunoblot experiments, Sertoli cell cultures were lysed in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of cell proteins (approximately 35 μg/lane) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and electro-blotted onto polyvinyledilene difluoride (PVDF) membranes. Protein loading and electroblotting efficiency were verified by Ponceau S staining, and the membrane was then blocked in Tween–Tris buffered saline (TTBS; 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin and incubated overnight with the primary antibody to be tested. The membrane was washed in TTBS and incubated with horseradish peroxidase-coupled anti-IgG antibody, washed again and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with the Opti-Quant® software. Blots were developed to be linear in the range used for densitometry.

2.4. Determination of intracellular reactive oxygen species

Intracellular ROS were detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA [38]). Briefly, DCFH-DA 10 μM was added to the cell culture 30 min before retinol incubation to allow cellular incorporation. Then, treatments were carried out as described above, the medium was discharged and cells were collected, resuspended in ice-cold PBS and sonicated. The DCFH-DA oxidation was quantified from the fluorescence emission intensity (model F2000, Hitachi Ltd., Tokyo, Japan) with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm.

2.5. Proliferation focus assessment

Cell focus assay was assessed as previously described [22]. Briefly, Sertoli cells were treated during 24 h with retinol 7 μM in the presence or absence of the

antioxidant Trolox® or PD98059 in the concentrations indicated above. The incubation medium was then replaced by Medium 199 pH 7.4 supplemented with 10% fetal bovine serum in all groups. Cells were maintained in humidified 5% CO_2 atmosphere at 34 °C for 14 to 15 days, with medium replacement every 3 days. Morphology was examined during this period and cell foci were scored at the end of the experiment under a light microscope.

2.6. Cell proliferation assay

As an index of cell proliferation we used the incorporation of [*methyl*-³H] thymidine, as previously described [39]. Briefly, after the first 24 h of culture in Medium 199 1% FSB, the medium was replaced for serum-free Medium 199 supplemented with 2.5 μCi/ml of [*methyl*-³H] thymidine (248 GBq mmol⁻¹; Amersham International, Amersham, UK). After 24 h, the medium was replaced by the same medium containing the drugs to be tested, and cells were incubated for more 24 h. Cells were then washed, harvested, suspended in nucleus isolation buffer (50 mM $NaPO_4$, 2 M NaCl, pH 7.4) and centrifuged (5 min, 14000×g) to extract nuclear DNA. An aliquot was used to determine [*methyl*-³H] thymidine incorporation into DNA in a Packard Tri-Carb Model 3320 scintillation counter, and another aliquot was utilized for DNA quantification by the propidium iodide and bisbenz-imidazole (Hoechst 33258) staining of isolated nuclear DNA, according to Labarca and Paigen [40].

2.7. Chemiluminescent assay for free radical quantification

To evaluate the cellular oxidant status we measured the *tert*-butyl hydroperoxide-initiated chemiluminescence. This method measures the balance between pro-oxidants and cell antioxidant defenses in the cellular environment. Briefly, cell homogenates were diluted to a final concentration of 1 mg/ml protein before adding *tert*-butyl hydroperoxide to a final concentration of 3 mM. Chemiluminescence was measured at 30 °C in a scintillation counter as previously described [41]. Values are expressed as percentage of counts/min/mg relative to the control cells.

2.8. Total radical-trapping antioxidant parameter (TRAP assay)

The antioxidant potential of cells was also estimated by the total radical-trapping antioxidant parameter (TRAP), which determines the non-enzymatic antioxidant potential of the cell. Briefly, the reaction was initiated by injecting luminol and AAPH (2,2′-Azobis[2-methylpropionamidine]dihydrochloride) – a free radical source that produces peroxyl radical at a constant rate – in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. After assays, Sertoli cell samples were suspended in glycine buffer, mixed into the reaction vial, and the decrease in luminescence, which is proportional to the non-enzymatic antioxidant potential, monitored in a liquid scintillation counter. The luminescence emission was followed for 60 min after the addition of the sample homogenates (150 μg of protein). Chemiluminescence values were standardized against protein content.

2.9. Measurement of mitochondrial superoxide production

Mitochondrial superoxide production was assessed as previously described [42]. To isolate submitochondrial particles (SMP) from Sertoli cell cultures, cells were homogenized in an isolation buffer (230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4). Homogenates were centrifuged (750×g, 10 min) to eliminate nuclei and cell debris and the pellet was washed to enrich supernatant; the supernatant was then centrifuged at 7000×g for 10 min. The pellet was washed and resuspended in the same buffer, and then SMP were obtained by freezing and thawing (three times) this fraction. The resulting SMP are washed twice with 140 mM KCl 20 mM Tris–HCl (pH 7.4) and resuspended in the same medium for determination of superoxide production, which was assessed by mixing SMP solution (0.3–0.1 mg protein/ml) to a reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl (pH 7.4), plus 0.1 mM catalase and 1 mM epinephrine. Succinate (7 mM) was used as substrate, and the superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (E480 nm 1/4 4.0/mM/cm) was followed by spectrophotometer. SOD was used at 0.1–0.3 mM final concentration to assess assay specificity.

2.10. Reduced/oxidized glutathione levels measurement and protein thiol content

Reduced (GSH) and oxidized (GSSG) glutathione were measured based on the same principle, according to the method of Griffith [43]. Briefly, cells were harvested in ice-cold phosphate buffer (0.1 M, EDTA 5 mM, pH 7.4) with 2.5% trichloroacetic acid (TCA), centrifuged (14000×g, 10 min) and the supernatant (100 μl) was added to a 1-ml cuvette containing 785 μl of phosphate buffer, 25 μl of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) 10 mM and 80 μl of NADPH 10 mM in order to determine GSH levels. After 3 min resting, the spectrophotometer at 412 nm was set blank with and the increase in absorbance was followed after the addition of glutathione reductase solution (750 mU/ml), and the values were compared to GSH standards measured by the same method. GSSG levels were measured by previously incubating both GSSG standards and a 100 μl aliquot of the same cell samples with 2-vinylpyridine for 60 min, and then following the same procedure for GSH determination. Total protein thiol content of cellular extracts was evaluated based on the same principle, by mixing total cell extracts (phosphate buffer without TCA) with DTNB (final concentration, 3 mM), reading samples at 412 nm and comparing values to a cysteine standard curve incubated with DTNB.

2.11. Protein quantification

Protein contents of each sample were measured as described by Lowry et al. [44] for data normalization.

2.12. Statistical analysis

Data are expressed as means±S.E.M. and were analyzed by one-way ANOVA followed by Duncan's post hoc test. Differences were considered to be significant at the $p < 0.05$ level.

3. Results

We have previously demonstrated that incubation of Sertoli cells with 7 μM retinol leads to cell cycle disruption and extensive cell damage through a process involving ROS generation. Thus, we first evaluated the ability of retinol to induce cellular free radicals production. Cells were loaded with the oxidant-sensitive fluorogenic probe dichlorodihydrofluorescein-diacetate (DCFH-DA) for 30 min before retinol incubation. This reagent is known to enter the cells, hydrolyze into dichlorodihydrofluorescein (DCFH), and, once inside the cells, it reacts predominantly with highly oxidizing species of ROS such as hydroxyl radicals (·OH) or hydroperoxides, thus producing the fluorophore dichlorofluorescein (DCF) [38]. Fig. 1A shows that retinol 5 μM does not increase basal ROS intracellular production during the first 15 min of incubation. However, treatment with retinol 7 μM is able to significantly increase ROS production, an effect abolished by the co-treatment with the general antioxidant Trolox (100 μM). Accordingly, the evaluation of the total non-enzymatic antioxidant capacity of Sertoli cells by the TRAP assay indicates that retinol 7 μM leads to an acute pro-oxidant pulse during the first 30 min of incubation, which is also reversed by Trolox co-treatment (Fig. 1B), suggesting that retinol leads to a partial consumption of cellular non-enzymatic antioxidants.

It is well described that mitochondrial electron transport chain is a cellular source of superoxide by the one-electron reduction of oxygen, and we recently reported that retinol increases superoxide production in isolated mitochondria, thus
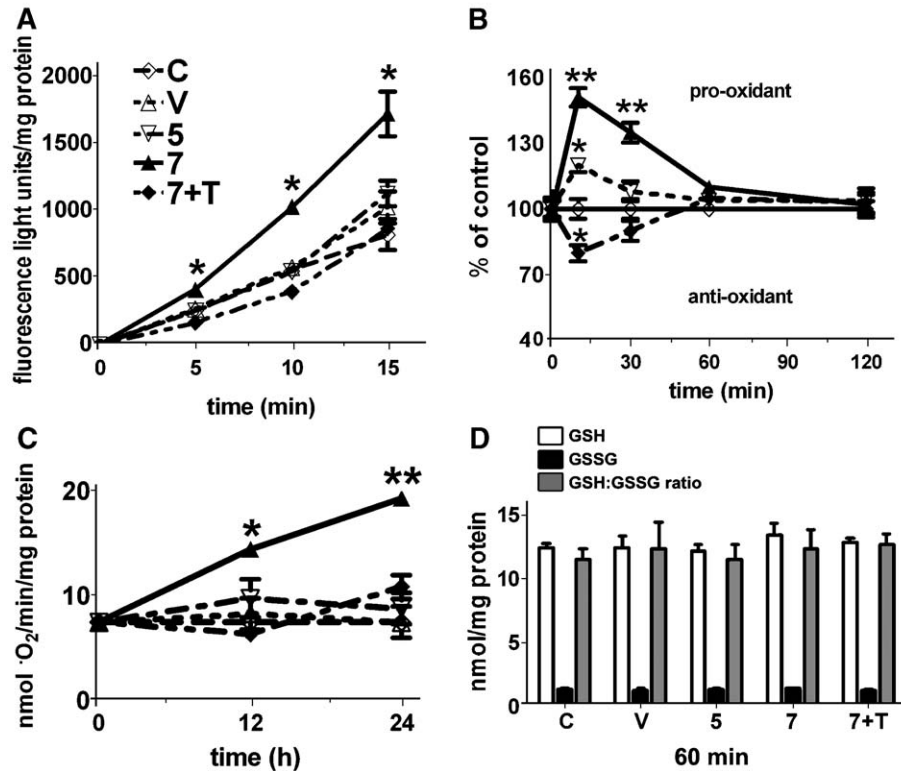
Fig. 1. Retinol induces cellular overall ROS production, causes a pro-oxidant pulse and increases mitochondrial superoxide production. Sertoli cells were incubated with retinol for 15 min and the production of ROS was assessed by the well established 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay (A). The non-enzymatic antioxidant potential of cells incubated with retinol was verified by the evaluation of the Total Radical-trapping Antioxidant Parameter (TRAP assay – B), and the mitochondrial superoxide production was evaluated by isolating submitochondrial particles from retinol-treated Sertoli cells (C). Both GSH and GSSG levels after a 60-min retinol incubation were quantified in total cellular extracts of cultures subjected to the same treatments, as described in Materials and methods (D). C=control, V=vehicle (0.1% ethanol v/v), 5=retinol 5 μM, 7=retinol 7 μM, 7+T=retinol 7 μM plus Trolox 100 μM. Data from three independent experiments, $n=3$; $*p<0.05$; $**p<0.01$.

leading to permeability transition and membrane swelling [23]. Therefore, we decided to examine whether retinol treatment affects mitochondrial superoxide production in Sertoli cells (Fig. 1C). In fact, retinol 7 μM caused a continuous increment in superoxide production during 24 h of incubation, and Trolox co-treatment inhibited this effect. Incubation with retinol 5 μM did not modify mitochondrial superoxide production, which agrees with our previous reports where concentrations below the physiological limit of 5 μM did not induce oxidative damage. On the other hand, both total glutathione levels, as well as the GSH/GSSG ratio, which are widely believed to be sensitive indicators of cell redox state, were not modified by retinol treatment (Fig. 2D), indicating that retinol-induced ROS did not affect thiol metabolism. In this regard, we evaluated if retinol treatment affected the total protein sulfhydryl cell content, which would be an indicative of oxidative modification of cysteine thiol groups from cell proteins, but no differences were detected (data not shown).

The ERK1/2 MAP kinases are known to be activated in a variety of situations where the cell redox state is modified by pro-oxidant agents, thus playing a key role in several ROS-induced changes in cellular metabolism and cell cycle regulation [45–47]. Therefore, to examine whether retinol stimulation is coupled to ERK1/2 activation in cultured Sertoli cells, cells were incubated with retinol (7 μM) for different

times and total cellular extracts were fractionated by SDS–PAGE and immunoblotted with an antibody able to specifically detect ERK1/2 only when dually phosphorylated (i.e. when activated; pERK1/2 [48]). Retinol induced a rapid and transient increase in pERK1/2 levels. This increase was already evident 1 min after the onset of incubation, peaked between 10 and 15 min, and lasted for at least 30 min (Fig. 2A); thus, we performed further experiments with cells incubated for 15 min. The increase in ERK1/2 phosphorylation was already detectable with retinol 5 μM, reached a plateau at 7 μM and was totally abolished by co-treatment with the antioxidant Trolox (Fig. 2B), therefore suggesting that the retinol-induced activation of ERK1/2 is indeed due to an increment in ROS production.

To better understand the mechanism by which retinol is causing the phosphorylation of ERK1/2, we co-treated Sertoli cells with retinol and different antioxidant agents (Fig. 2C). Besides Trolox, the ·OH radical scavenger mannitol (1 mM) reversed retinol-induced ERK1/2 phosphorylation; the addition of native superoxide dismutase (SOD, EC 1.12.1.1, 200 U/ml) in the incubation medium together with retinol had a similar effect. Since these data suggested the involvement of both superoxide and ·OH radicals in the effect of retinol upon ERK1/2 phosphorylation – which, in turn, suggests the occurrence of transition metal-catalyzed Fenton reaction – we examined the effect of the iron chelator 1,10-phenanthroline (100 μM), which
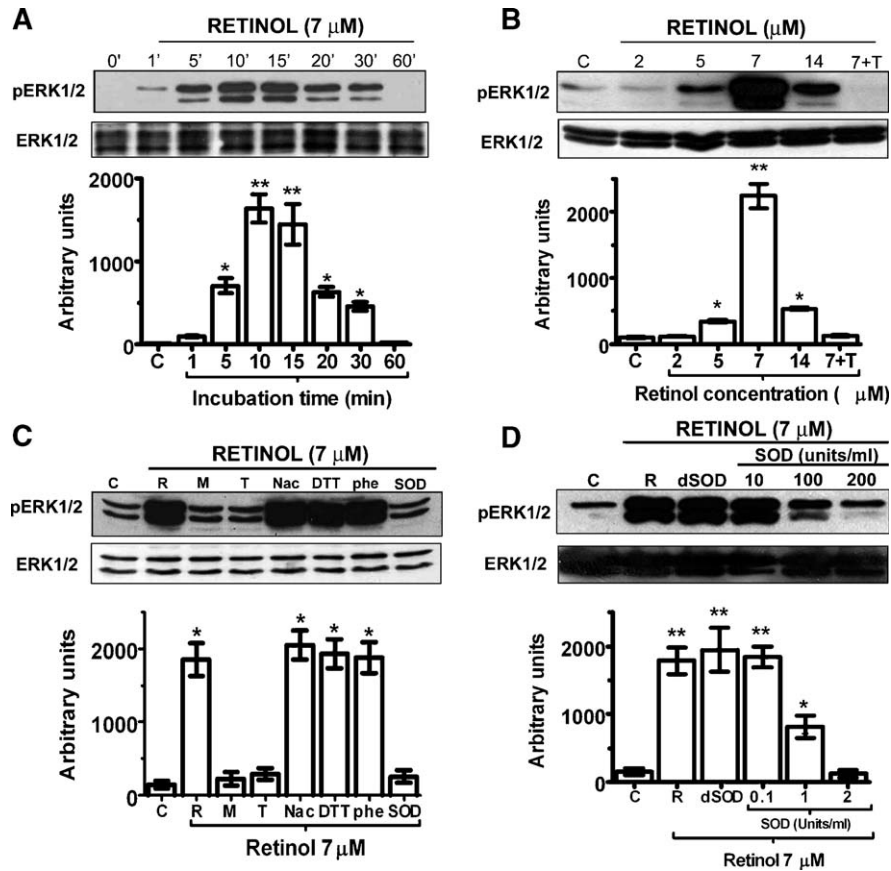
Fig. 2. Retinol-induced ERK1/2 phosphorylation is reversed by antioxidant treatment. Representative immunoblots with respective densitometric analyses (means±S.E.M.) showing the time-course (A) of retinol-induced phosphorylation of ERK1/2 (pERK1/2) in cultured Sertoli cells; incubation of cells for 15 min (B) with different retinol concentrations demonstrated a dose-dependent response, and the reversion retinol-induced phosphorylation by Trolox co-treatment (7+T=retinol 7 μM plus Trolox 100 μM). The blot in panel C shows the effect of different antioxidants co-treatments with retinol on ERK1/2 phosphorylation: mannitol 1 mM (M), Trolox 100 μM (T) and native superoxide dismutase enzyme 200 U/ml (SOD) co-incubation reversed retinol-induced phosphorylation, while N-acetyl-cysteine 1 mM (Nac), dithiothreitol 1 mM (DTT) and the iron chelator 1,10-phenantroline 100 μM (phe) did not exert any effect. 200 U/ml of denatured SOD (dSOD) was used as a control for SOD treatment (D), which was demonstrated to be more effective in reversing the effect of retinol at the higher concentration tested (200 U/ml). $*p<0.05$; $**p<0.01$; $**p<0.005$; $n=3$, triplicate.

we previously observed to inhibit retinol-induced oxidative damage in Sertoli cells [16–20,22]. However, 1,10-phenantroline co-treatment did not show any effect upon retinol-induced ERK1/2 phosphorylation (Fig. 2C). Also, we tested the thiol antioxidants dithiothreitol (DTT; 1 mM), which protects protein thiol groups from oxidation, and N-acetyl-cysteine (NAC, 1 mM), which acts as a thiol antioxidant per se and also stimulates GSH synthesis, since it has been reported that some MAPK family members and/or upstream activator kinases can be activated by oxidative modification of sulfhydryl groups from some of their cysteine residues [49]. Nonetheless, both DTT and NAC did not show any effect upon retinol-evoked ERK1/2 phosphorylation, which suggests that although retinol is causing the phosphorylation of ERK1/2 by a ROS-mediated mechanism, protein thiol oxidation appears not to be involved in this effect. Addition of denatured SOD (200 U/ml, previously boiled for 5 min to destroy SOD activity), a control for SOD treatment, did not affect retinol-evoked ERK1/2 phosphorylation, and concentrations of native SOD below 200 U/ml did not demonstrate to be effective in inhibiting this effect (Fig. 2D).

The Src family of tyrosine kinases is one of the earlier effectors of ROS-induced cell damage [50] and several evidences indicate that these kinases control the activation state of the ERK1/2 pathway in different experimental systems through the stimulation of the MAPK/ERK kinases 1/2 (MEK1/2) [51]. Thus, to elucidate whether retinol activates ERK1/2 through upstream Src–MEK1/2 pathway, we co-treated Sertoli cells with retinol and PP2 (10 μM), a well-described Src family inhibitor, or with the MEK1/2 inhibitor PD98059 (10 μM). The phosphorylation of ERK1/2 by retinol was inhibited by both PP2 and PD98059 (Fig. 3A), suggesting that Src tyrosine kinase and MEK1/2 are involved in the control of retinol-induced phosphorylation of ERK1/2. To confirm that, we further examined whether retinol treatment activates Src and MEK1/2, and we found that retinol 7 μM indeed induces Src and MEK1/2 phosphorylation (Fig. 3B). Trolox, mannitol and native SOD also inhibited this effect, thus suggesting that retinol-induced increase in cellular ROS production is causing this effect. Once again, thiol antioxidants (NAC, DTT) and the iron chelator 1,10-phenantroline did not reverse retinol-evoked phosphorylation of both Src and MEK1/2. Addition of SOD
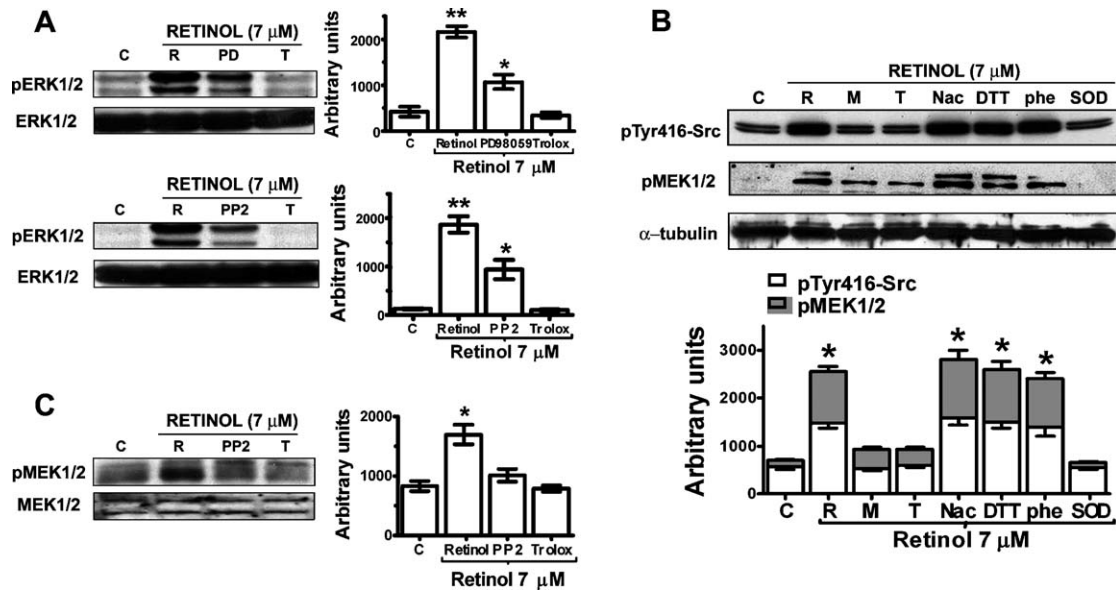
Fig. 3. Retinol-induced ERK1/2 phosphorylation is Src- and MEK1/2-dependent. Representative immunoblots and respective densitometric analyses (means±S.E.M.) showing the inhibition of retinol-induced ERK1/2 phosphorylation by the MEK1/2 inhibitor PD98059 10 μM and the Src inhibitor PP2 10 μM, comparing to the inhibitory effect of Trolox μM (A). To further confirm the involvement of Src and MEK1/2 in retinol-induced ERK1/2 phosphorylation, we directly examined Src and MEK phosphorylation levels in cells treated with retinol 7 μM (B), and the effects of mannitol 1 mM (M), Trolox 100 μM (T), N-acetyl-cysteine 1 mM (Nac), dithiothreitol 1 mM (DTT), 1,10-phenantroline 100 μM (phe) and native superoxide dismutase enzyme 200 U/ml (SOD) co-incubation; constitutive α-tubulin was used as loading control. MEK1/2 phosphorylation is also inhibited by PP2 co-treatment (C), thus confirming the upstream role of Src tyrosine kinase. C=control, R=retinol, PD=PD98059. * $p < 0.05$; ** $p < 0.01$; $n = 3$.

without retinol, or co-treatment of retinol and denatured SOD (dSOD) did not show any effect either (not shown). Finally, to determine if retinol-induced Src and MEK1/2 phosphorylation are coupled events, we verified the effect of the Src inhibitor PP2 on MEK1/2 phosphorylation (Fig. 3C). PP2 co-treatment inhibited retinol-induced MEK1/2 phosphorylation, which confirmed that the ERK-activating kinases MEK1/2 are phosphorylated by a Src-dependent pathway in Sertoli cells treated with retinol.

One of the major downstream targets of ERK1/2 is the transcription factor CREB, which has been reported to trigger the ROS-induced ERK1/2-mediated activation of several genes. Once activated by phosphorylation at Ser-133, CREB (pCREB) modulates cell cycle/apoptosis regulation genes, and also other transcription factors, such as different members of the AP-1 family, among others [26,27]. Thus, we evaluated the ability of retinol to induce CREB phosphorylation in Sertoli cell cultures. As can be seen in Fig. 4A, retinol produced a clear-cut increase in pCREB levels which followed a similar time course as the observed for ERK1/2 phosphorylation. The phosphorylation of CREB brought on by retinol reached a plateau at the concentration of 7 μM, being reversed by Trolox co-treatment (Fig. 4B), and the different antioxidant treatments presented the same pattern of inhibition observed for the upstream Src–MEK1/2–ERK1/2 kinases (Fig. 4C). Also, it is important to note that CREB phosphorylation was completely blocked by both the MEK inhibitor PD98059 (10 μM, Fig. 4D) and the Src inhibitor PP2 (10 μM, Fig. 4E), which confirmed the involvement of these kinases in retinol-induced CREB phosphorylation.

It has been suggested that downstream effectors of ERK1/2, such as CREB, could be involved in some of the aspects associated with cellular proliferation and malignant transformation induced by ROS [26–28,31–36]. Since we have previously shown that retinol leads to cell proliferation, proliferative focus formation and morphologic transformation by increasing ROS production in Sertoli cells [22,39], we decided to study whether retinol-induced ERK1/2 phosphorylation is involved in these processes. In this regard, we verified whether the MEK1/2–ERK1/2 inhibitor PD98059 is able to block the increase in DNA synthesis and [*methyl*-³H] thymidine incorporation induced by 24 h treatment with retinol, as well as the induction of proliferative focus formation in cells cultured for 14 days. Since we previously observed that antioxidant co-treatment reverses the effect of retinol upon these parameters [16–23], we compared the effect of the MEK1/2–ERK1/2 inhibitor with the effect of Trolox. We found that PD98059 abolished the effect of retinol 7 μM on morphologic transformation and proliferative focus formation (Fig. 5A and B), and that the retinol-induced increase in DNA synthesis (Fig. 5A, top table) and [*methyl*-³H] thymidine incorporation (Fig. 5B) after 24 h of incubation were also reversed by the co-treatment with the MEK inhibitor, in a similar effect to that previously observed with the co-administration of Trolox and other antioxidants [22,39]. To ensure that these results were not caused by a possible reduction in cellular ROS production caused by PD98059 administration, we measured the oxidant cellular status 24 h after the onset of the co-incubation with retinol and the MEK inhibitor by the quantification of the *tert*-butyl hydroperoxide-initiated chemiluminescence (Fig. 5C).
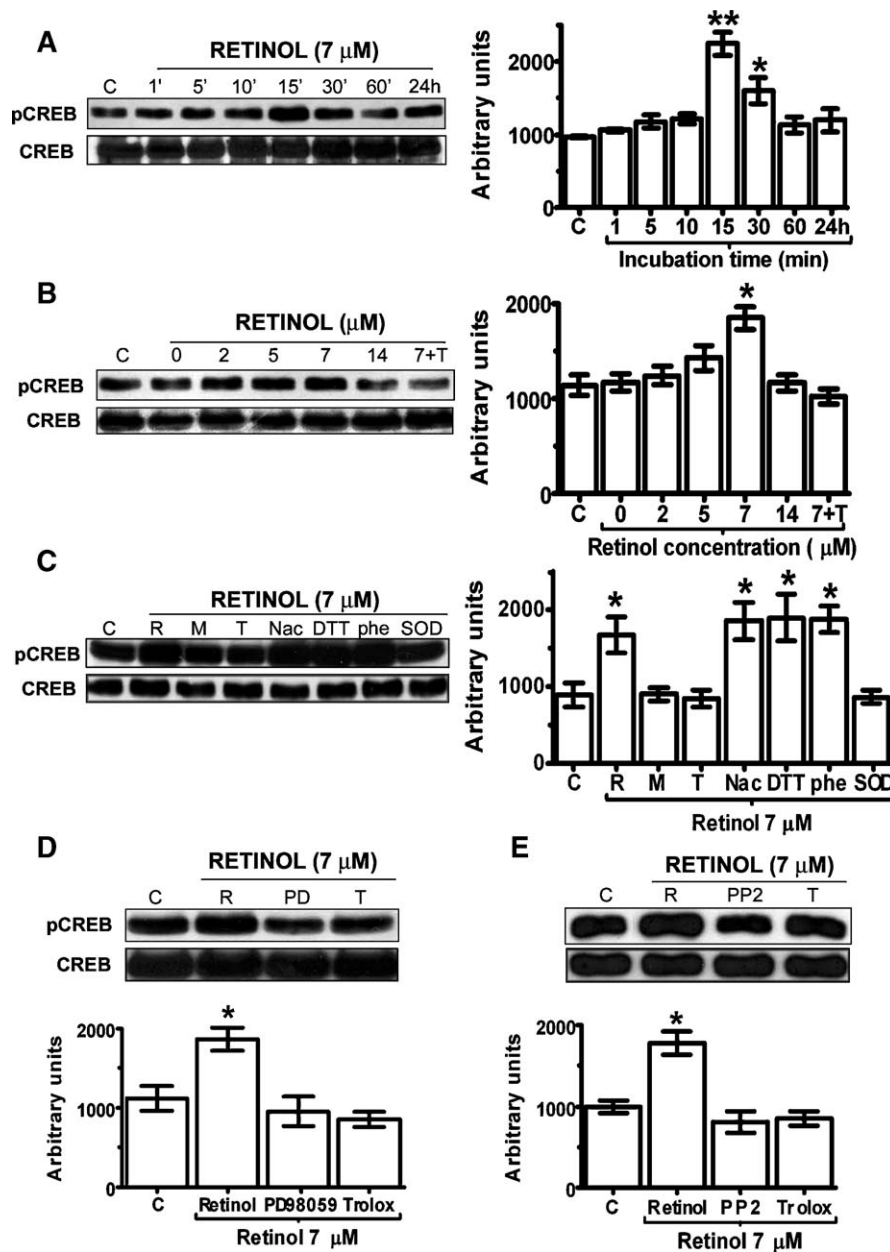
Fig. 4. Retinol-induced CREB phosphorylation is reversed by antioxidant treatment and is casually related to Src and ERK1/2 activation. Representative immunoblots with respective densitometric analyses (means±S.E.M.) showing (A) time-course and (B) dose-dependent effect (15 min of incubation; C=control, 0=vehicle and 7 +T=retinol 7 μM plus Trolox 100 μM) of retinol treatment leading to CREB phosphorylation (pCREB). Antioxidants Trolox 100 μM (T), mannitol 1 mM (M), and native superoxide dismutase 200 U/ml (SOD) reversed the effect of retinol, while Nac 1 mM, DTT 1 mM and 1,10-phenantroline 100 μM did not exert any effect (C). Inhibition of ERK1/2 phosphorylation by PD98059 10 μM (PD; D) or Src phosphorylation by PP2 (E) showed a similar inhibitory effect to that caused by the anti-oxidant Trolox 100 μM (T). $*p < 0.05$; $**p < 0.01$; $n = 3$.

Our results demonstrated that despite the co-administration of Trolox 100 μM suppress the increase in cellular ROS production caused by retinol treatment, PD98059 10 μM co-incubation did not interfere in retinol-increased ROS production.

4. Discussion

We and others have postulated that retinol might induce oxidative stress in different cell systems [16–23,39,52]. Although vitamin A is accepted as essential for various vital cellular processes, the effects of retinol supplementation are still poorly understood, and its ability to act as a pro-oxidant factor has been neglected until recent years. Works from our group have indeed demonstrated that retinol can be a pro-oxidative and thus deleterious molecule even at concentrations (7 μM) only slightly above the cellular physiologic range reported for Sertoli cells (which ranges between 2 and 5 μM depending on the feeding state and plasma retinol levels [53]). Sertoli cells constitute a physiological target of retinol, which regulates many of the reproductive-related paracrine functions of these cells by an intricate signaling process [53,54]. Thus, the
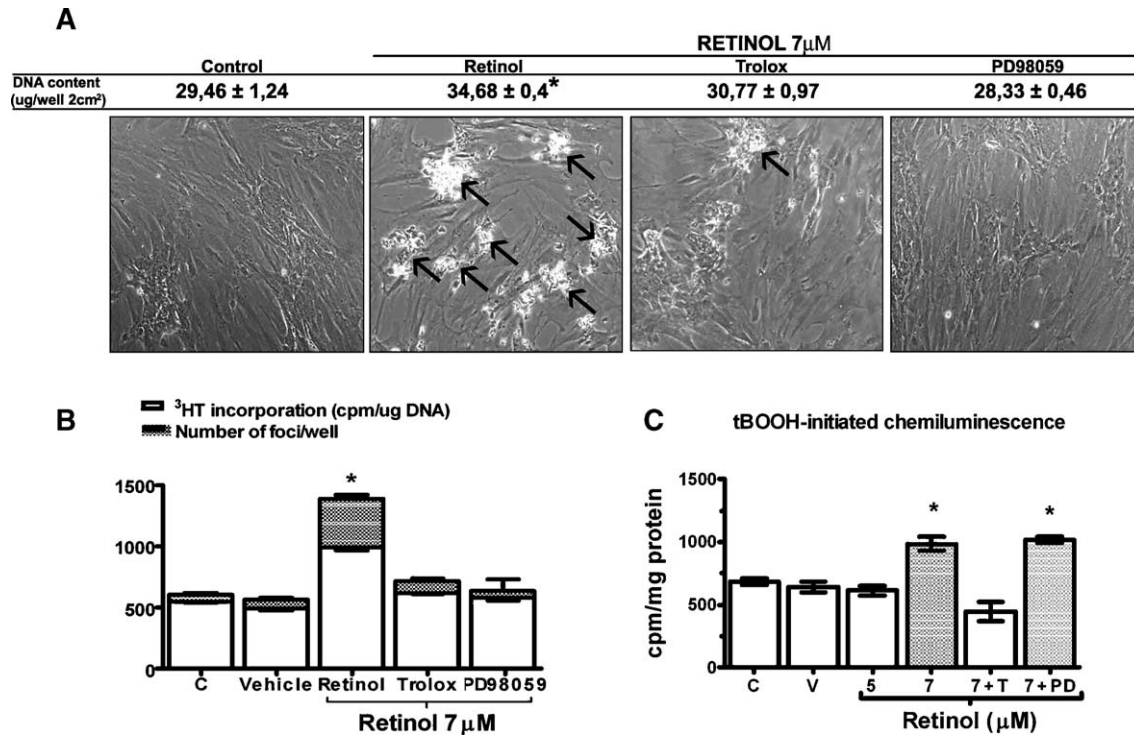
Fig. 5. PD98059 treatment inhibits retinol-induced proliferative focus formation, increased DNA synthesis and augmented incorporation of [*methyl*-³H] thymidine mediated by ROS production in Sertoli cells. Sertoli cells were treated for 24 hours with retinol and the effect of ERK1/2 inhibition by PD98059 10 μM co-administration on several parameters of proliferation and cell transformation was analyzed. (A) Total DNA content (means±S.E.M.) evaluated by Hoescht/PI fluorescence assay (top panel) and morphologic analysis of proliferative foci (identified by arrows) appearance after 14 days of retinol treatment (representative photomicrography, 40×). (B) Incorporation of [*methyl*-³H] thymidine and number of proliferative foci/dish of 24-well multiplate in Sertoli cells treated for 24 with retinol and PD98059. (C) PD98059 incubation for 24 h did not inhibit ROS production by retinol, as assessed by *tert*-butyl hydroperoxide (tBOOH)-initiated chemiluminescent assay, thus confirming that the effect observed above is due to ERK1/2 inhibition. C=control, V=vehicle (ethanol 0.1% v/v), T=Trolox 100 μM, PD=PD98059 10 μM. ∗$p < 0.05$; data from three independent experiments, $n = 3$.

relevance of the results presented in this work lies not only on the effects caused by retinol per se, but also in the demonstration that slight fluctuations in its concentration can determine abrupt modifications in the cellular redox status, and, consequently, to cause the switch of the cell cycle from a non-proliferative status to a proliferative, apoptotic or even a transformed condition.

In this work, we also demonstrated that retinol treatment enhances overall ROS production in short times, thus causing a pro-oxidant pulse in cultured Sertoli cells, which seems not to be due to a decrease in GSH levels nor modifications in the GSH/GSSG ratio. On the other hand, our present data demonstrating increased mitochondrial superoxide production, together with our recent observations that retinol induces ROS-dependent opening of mitochondrial permeability transition pore with consequent cytochrome *c* release [23], suggest that the main mechanism by which retinol leads Sertoli cells to oxidative stress is through mitochondrial superoxide production. Corroborating with these observations, our previously reported data showing the increase in catalase, GSH-Peroxidase and SOD activities induced by retinol, as well as the induction of lipoperoxidation and DNA oxidative damage by an iron-dependent mechanism [18–20,22], indicate that retinol is indeed able to enhance mitochondrial superoxide formation, thus increasing H_2O_2 production through SOD activity, which, in the presence of iron, may lead to DNA oxidative damage

through Fenton chemistry-dependent ·OH radical production after a 24 h treatment. Alterations in the mitogenic state and/or cell survival status may be consequences of such oxidative injuries after long-term exposition to retinol, as we have previously observed [16,17,22], combined with oxidative-dependent modifications in the activation state of kinase pathways regulating mitogenic cycle/senescence, such as the Src–MEK1/2–ERK1/2–CREB pathway.

Here we report that retinol is able to induce the ROS-mediated activation of ERK1/2 in a Src- and MEK1/2-dependent manner, which leads to the activation of the transcription factor CREB. Src and ERK participate in this pathway since inhibition of these kinases reverses CREB phosphorylation. Nevertheless, the activation of this kinase phosphorylation cascade seems to be casually related to the increased production of ROS caused by retinol. We observed that the general antioxidant Trolox, a hydrophilic analogue of vitamin E, inhibits oxidative-related effects of retinol upon Sertoli cells, such as the increase in lipoperoxidation, mitochondrial superoxide production, DNA oxidative damage and cellular ROS generation. In this regard, we also observed that Trolox co-treatment abolished the effect of retinol upon the kinases studied, which strongly indicates that ERK1/2 and CREB phosphorylation is induced by increased ROS production. The ·OH radical scavenger mannitol reversed ERK1/2 and

CREB phosphorylation, an effect also observed with the simple addition of native SOD in the incubation medium – in accordance with our previous studies, where both mannitol and native SOD inhibited lipoperoxidation and protein oxidative damage caused by 24 h treatment of retinol 7 μM. Taken together, these data reinforces the involvement of both superoxide and hydroxyl radicals on the effect of retinol treatment upon Sertoli cells, therefore indicating the existence of a causal link between the retinol-promoted increase in ROS production and the activation of the Src–MEK1/2–ERK1/2–CREB phosphorylation pathway. Interestingly, both NAC and DTT co-treatments demonstrated to be ineffective in reversing the effects of retinol upon Src–MEK1/2–ERK1/2–CREB pathway, suggesting that oxidative modification of sulfhydryl groups from cysteine residues is not the mechanism by which these kinases and the transcription factor are activated during retinol-induced oxidative stress. In this sense, the inability of the iron chelator 1,10-phenantroline in inhibiting ERK1/2–CREB phosphorylation indicates that the activation of these kinases is not dependent on iron-catalyzed production of hydroxyl radicals. Since we observed that mannitol – a classical hydroxyl scavenger – inhibited kinase activation, other pathways for $^{.}OH$ radicals production during the first 15 min of retinol treatment may account for this effect than Fenton, e.g. Haber–Weiss reactions (which sequentially utilizes superoxide, H_2O_2 and H^+ to produce $^{.}OH$) and other iron-independent pathways.

The ERK1/2–CREB signaling pathway is commonly associated with the regulation of cell cycle, by suppressing/stimulating proliferation and cell cycle arrest [55,56]. Here, we demonstrate that ERK1/2 phosphorylation is a necessary step for the oxidant-dependent cell transformation and proliferative focus formation caused by retinol. It is interesting to note that PD98059 completely reversed the morphologic transformation and other proliferative parameters without affecting the retinol-induced increase in ROS production, as demonstrated by the chemiluminescent quantification of free radicals production. This suggests that the MAPK/CREB pathway is an essential step for the mitogenic/transformative signaling mediated by oxidants produced during retinol treatment, reinforcing the importance of this signaling pathway in the activation/regulation of the cell cycle in a non-proliferative and terminally differentiated type of cell, which is the case of the primary-cultured Sertoli cells.

In our previous works, we have demonstrated that retinol activates ornithine decarboxylase [18], causes HMG dephosphorylation, and induces phosphorylation of the H3 histone [17], which are important markers of entry into the S phase of the cell cycle [57]. It is known that the p90Rsk-family member Rsk2, an ERK1/2-activated ribosomal S6 kinase, which is the mediator of the ERK1/2-induced activation of CREB [27,58], induces H3 phosphorylation, thereby regulating gene transcription during transformation [59,60]. As our data indicate that ERK1/2 activation is required for retinol-induced cell proliferation and transformation to occur, it seems well conceivable that phosphorylation of these kinases together with the previously observed retinol-induced histone phosphorylation could be involved in DNA synthesis activation during proliferative and transformative processes, as it has been observed in different cell models [61,62].

Recently, Cañon et al., suggested that the retinoic acid nuclear receptors (RAR), upon activation by retinoic acid (RA), could act directly on ERK1/2, causing the phosphorylation of these kinases and consequently the activation of CREB [63]. Based on their observations of this rapid response of CREB/ERK phosphorylation to RA, they suggested that RA, by acting on RAR, is able to exert rapid and non-genomic effects, not compatible with the well-established RAR-mediated transcriptional effects of this retinoid. Our results are in accordance with a rapid, non-genomic effect possibly exerted by retinol, and although we can not determine whether, in our model, retinol is or is not metabolized to RA, our data support the hypothesis that non-genomic effects caused by retinol in Sertoli cells are dependent on its ability to enhance cellular ROS production, which may affect many redox-sensitive signaling pathways in this and possibly other cellular models. The consequences of redox-sensitive signaling pathways activation may vary in different cell types but, at least in Sertoli cells, it appears to be tightly coupled to cell cycle regulation and cell transformation.

In conclusion, our results show that retinol, by enhancing free radicals' production, leads to Src, MEK1/2, ERK1/2 and CREB phosphorylation. The activation of this pathway appears to be involved in the onset of some of the deleterious effects caused by retinol supplementation in Sertoli cells, including malignant transformation and cell proliferation, and the data presented in this work may thus contribute to a better understanding of the redox-related properties of this vitamin upon biological systems.

Acknowledgements

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Capítulo III.

Retinol activates tyrosine hydroxylase acutely by increasing the phosphorylation of serine 40 and then serine 31 in bovine adrenal chromaffin cells

Retinol activates tyrosine hydroxylase acutely by increasing the phosphorylation of serine40 and then serine31 in bovine adrenal chromaffin cells

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Abstract

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of the catecholamines. It has been reported that retinol (vitamin A) modulates tyrosine hydroxylase activity by increasing its expression through the activation of the nuclear retinoid receptors. In this study, we observed that retinol also leads to an acute activation of tyrosine hydroxylase in bovine adrenal chromaffin cells and this was shown to occur via two distinct non-genomic mechanisms. In the first mechanism, retinol induced an influx in extracellular calcium, activation of protein kinase C and serine40 phosphorylation, leading to tyrosine hydroxylase activation within 15 min. This effect then declined over time. The retinol-induced rise in intracellular calcium then led to a second slower mechanism; this involved an increase in reactive oxygen species, activation of extracellular signal-regulated kinase 1/2 and serine31 phosphorylation and the maintenance of tyrosine hydroxylase activation for up to 2 h. No effects were observed with retinoic acid. These results show that retinol activates tyrosine hydroxylase via two sequential non-genomic mechanisms, which have not previously been characterized. These mechanisms are likely to operate *in vivo* to facilitate the stress response, especially when vitamin supplements are taken or when retinol is used as a therapeutic agent.

Keywords: adrenal chromaffin cells, extracellular calcium, non-genomic effects, protein phosphorylation, retinol, tyrosine hydroxylase activation.

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Retinol (vitamin A) and its metabolites (collectively known as retinoids) are involved in the regulation of diverse physiological functions, such as vision, reproduction and growth. Classical effects of retinol are mediated by the activation of the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), which are transcription factors belonging to the steroid-thyroid-retinoid hormone receptor super family (Chambon 1994). Activation of these receptors induces binding to retinoid response elements and initiates gene transcription, which is known as the 'genomic' or 'nuclear' action of retinol. More recently, a second mechanism of action has been described for retinoids, not involving RAR/RXR-mediated gene transcription. This has been referred to as the 'non-genomic' or 'extra-nuclear' effect of retinoids. These non-genomic effects of retinoids are mediated by the activation of second messenger pathways and they lead to rapid physiological changes, including increased synaptic transmission (Liao *et al.* 2004; Pasquali *et al.* 2005; Aggarwal *et al.* 2006; Gelain *et al.* 2006).

Tyrosine hydroxylase (TH, EC1.14.16.2) is the rate-limiting enzyme in the catecholamine synthesis pathway, and its regulation controls the levels of dopamine, epinephrine and norepinephrine in tissues. To compensate for catecholamine loss as a result of secretion, TH activity is physiologically up-regulated by two mechanisms: increased protein expression, or increased protein phosphorylation. Phosphorylation of serine (ser) 40 releases TH from feedback inhibition by catecholamines, and is the main mechanism for short-term TH activation, increasing enzyme activity up to 20-fold (Daubner *et al.* 1992; Dunkley *et al.* 2004). Phosphorylation of TH at ser31 and ser19 can also increase TH

activity; however, the contribution of each site is much smaller than that of ser40. Ser31 phosphorylation can induce a twofold increase in TH activity by altering the enzymes' affinity for the cofactor tetrahydrobiopterin (BH4) (Dunkley *et al.* 2004). Phosphorylation of ser31 can also facilitate ser40 phosphorylation by hierarchical phosphorylation, which leads to TH activation *in situ* (Dunkley *et al.* 2004; Lehmann *et al.* 2006). Ser19 phosphorylation does not affect TH activity directly, but it can facilitate ser40 phosphorylation by binding to the 14 : 3 : 3 protein, or by hierarchical phosphorylation (Bevilaqua *et al.* 2001; Toska *et al.* 2002; Bobrovskaya *et al.* 2004; Dunkley *et al.* 2004).

There is a growing body of evidence implicating retinoids as important regulators of catecholamine cell function. Retinoic acid (RA), the most active metabolite of retinol, is classically described as an *in vitro* inducer of differentiation of catecholaminergic cell types, such as the SK-N-BE(2)C neuroblastoma and the TH-expressing PC12 cell line (Pahlman *et al.* 1984; Matsuoka *et al.* 1989; Lee and Kim 2004). TH expression is increased by RA in a range of cells (Cosgaya *et al.* 1996; Cheung *et al.* 2000; Zhang *et al.* 2006a) and RAR directly transactivate the *TH* gene by binding to a RARE sequence in the promoter region (Jeong *et al.* 2006). However, acute effects of retinoids on TH have not been investigated.

In this study, we investigated whether retinol and RA could influence TH activation by non-genomic mechanisms. We used bovine adrenal chromaffin cells (BACC) and we analyzed the effects of incubating these cells with retinol or RA for up to 2 h on the activity and the phosphorylation of TH. We found that retinol activated TH by increasing ser40 and ser31 phosphorylation, but RA had no effects. We then investigated the signal transduction pathways that led to the increases in TH phosphorylation by applying protein kinase inhibitors and determining the levels of protein kinase activation and second messenger levels in the BACC. The results show for the first time that TH activation can be induced by two distinct non-genomic actions of retinol.

Material and methods

Chemicals

EGTA, *N*-acetyl-cysteine (NAC), dithiothreitol (DTT), fura-2AM, 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxy-methyl) ester (BAPTA-AM), pluronic F-127, all-*trans*-retinol alcohol, all-*trans*-retinoic acid (RA), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethyl)-2,5- diphenyl tetrazolium bromide (MTT), nicotine, Tween-20, and β-mercaptoethanol were from Sigma Chemical Co. (St Louis, MO, USA). Protease inhibitor tablets (complete) were from Roche Molecular Biochemicals (Indianapolis, IN, USA). BH4 was from Dr B. Schircks (Jona, Switzerland). Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA), M molecular-weight PAGE

standards, nitrocellulose membrane (Hybond ECL), enhanced chemiluminescence kit (ECL plus), anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) and L-[3–5-3H]tyrosine (1 mCi/mL) were from Amersham Pharmacia Biotech (Amersham, UK). Phosphorylated serine (pser)40TH-, pser31TH- and pser19TH-specific antibodies were generated and tested for specificity as described by Cammarota *et al.* (2003) and were also obtained from Sigma Chemical Co. A pser protein kinase C (PKC) substrate antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Phospho-extracellular signal-regulated kinases 1/2 (ERK 1/2) specific antibody was from Zymed Laboratories Inc. (San Francisco, CA, USA). UO126 was from Promega Corporation (Madison, WI, USA), GÖ6983 and SB203580 were from Merck Biosciences (Darmstadt, Germany) and H89 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All inhibitors were dissolved in dimethyl sulfoxide (DMSO). Retinol and RA were dissolved in ethanol. Concentrated stocks were prepared immediately before experiments by diluting retinol or RA into ethanol and determining final stock concentration by UV absorption; solutions were kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition. The final DMSO or ethanol concentration did not exceed 0.2% in any experiment. Tissue culture reagents were from Sigma Chemical Co. and were of analytical or tissue culture grade.

Bovine adrenal chromaffin cells isolation, culture and treatments

Bovine adrenal chromaffin cells were isolated from adrenal glands by sequential protease and collagenase digestion in a shaking waterbath followed by enrichment for chromaffin cells by density-gradient centrifugation through 4% bovine serum albumin (BSA) as described by Zerbes *et al.* (1998). Cells were plated onto plastic culture plates at a density of 3×10^5 cells/cm^2 and maintained under a 5% CO$_2$-containing atmosphere at 37°C in the presence of complete medium (Dulbecco's modified Eagle's medium supplemented with 4 mmol/L L-glutamine, 5.5 mmol/L glucose, 10% fetal calf serum (FCS), 15 mmol/L HEPES, pH 7.4, 100 mg/mL penicillin G, 100 mg/mL streptomycin, 50 mg/mL gentamicin and 5 mg/mL mycostatin for 4–7 days. Medium was changed every 3–4 days. Immediately before treatment, cells were washed once with sterile phosphate-buffered saline and then pre-treated for 1.5 h with serum-free medium (1 mL/well, complete medium without addition of FCS). Antioxidants (NAC, 1 mmol/L and DTT, 1 mmol/L), EGTA (4 mmol/L) and kinases inhibitors (UO126 10 μmol/L, GÖ6983 1 μmol/L, H89 10 μmol/L, KT 5823 300 nmol/L and SB203580 1 μmol/L) when used, were added to serum-free medium 30 min prior to retinol treatment. Retinol and RA treatments were initiated by adding concentrated solutions to reach final concentrations in the well. Treated cells were then used to analyze protein phosphorylation and TH activity. MTT viability assays indicated that cell viability was not affected during incubations (not shown). For determination of intracellular calcium changes, retinol treatment was carried out using a perfusion system, as described in 'calcium measurements' section.

Protein phosphorylation

Western blot assays were performed to determine TH phosphorylation at ser40, ser31, and ser19, to detect the phosphorylated (i.e.

active) form of ERK 1/2, and to determine the activation state of PKC by detecting phosphorylated PKC substrates. For these measurements, BACC were plated onto 24-well plates and treatments were terminated by adding Laemmli-sample buffer to BACC (final concentrations 50 mmol/L Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2 mmol/L EDTA) and proteins were fractioned by SDS-PAGE and electroblotted onto nitrocellulose membranes. Protein loading and electroblotting efficiency were verified by Ponceau S staining, and membranes were washed and blocked using Tris-buffered saline with Tween (100 mmol/L Tris–HCl, 0.9% NaCl, 0,1% Tween-20, pH 7.5). Membranes were incubated for 1 h at room temperatures (25°C) with the primary antibodies to be tested at the concentrations suggested by the manufacturer, or as previously described (Bobrovskaya *et al.* 2007a, b). Membranes were washed and incubated with horseradish peroxidase-linked anti-IgG antibody, and immunoblots were visualized and quantitated on the Typhoon (Molecular Dynamics, Sunnyvale, CA, USA) imaging system using ECL- plus detection reagents. The density of pser19 TH, pser 31TH, pser40 TH, pERK 1/2 and PKC-phosphorylated protein bands (all bands were included) was measured and expressed as a fold increase of the mean of the control samples (unstimulated cells incubated with buffer alone were assigned a level of expression of onefold). Loading control blots for total proteins (total TH and total ERK 1/2) at 2 h of incubation were performed to confirm that there were no changes in the expression pattern of these proteins.

TH activity

Bovine adrenal chromaffin cells were plated on to 12-well culture plates at a density of 2.5×10^6 chromaffin cells/well and then treated. After treatments the medium was aspirated and 0.175 mL ice-cold 50 mmol/L Tris (pH 7.4) containing 1 mmol/L EGTA, 1 mmol/L EDTA, 1 µmol/L microcystin, 80 µmol/L ammonium molybdate, 2 mmol/L tetrasodium pyrophosphate, 1 mmol/L DTT, 1 mmol/L sodium vanadate, 1 mmol/L β-glycerophosphate and protease inhibitor cocktail (complete, 1 tablet) was added to the cells. The cells were scraped and lysed by passing through a 26-G needle (Terumo Corporation, Tokyo, Japan). The lysate was centrifuged at 15 000 *g* for 15 min at 4ºC. The supernatant was transferred to ice and then immediately assayed for TH activity. TH activity was measured using a method based on the tritiated water release assay (Reinhard *et al.* 1986). The reaction mixture contained 65 µg/mL catalase, 50 mmol/L potassium phosphate (pH 7.2), 2 mmol/L β-mercaptoethanol, 25 µmol/L tyrosine (including [³H] tyrosine) and 2 mmol/L BH4. TH activity was measured at 25ºC for 9 min. The assay was linear over this time. The changes in TH activity was expressed as a fold-increase in relation to the mean of the control samples (cells incubated with buffer alone; onefold).

Reactive oxygen species

Intracellular reactive oxygen species (ROS) production was determined by the DCFH-DA-based real-time assay using intact living cells, as described by Wang and Joseph (1999). In brief, BACC were plated onto 96-well plates and incubated for 1 h with DCFH-DA 100 µmol/L (stock solution in DMSO, 10 mmol/L) in 1% FCS culture medium at 5% CO_2 and 37ºC. Later, cells were washed and treatments were carried out. During treatment, changes in the fluorescence by the oxidation of DCFH into the fluorogen DCF were

followed in a microplate fluorescence reader (FLUOstar Optima, BMG LabTechnologies, Offenburg, Germany) for 1 h at 37ºC. H_2O_2 5 mmol/L was used as positive control for fluorescence. Excitation filter was set at 485 ± 10 nm and the emission filter was set at 530 ± 12.5 nm. Data were recorded every 30 s by Fluostar Optima 1.30 R3 software and plotted in Excel.

Intracellular calcium ($[Ca^{2+}]_i$)

Determination of changes in $[Ca^{2+}]_i$ was made by measurement of fura-2AM fluorescence intensity. BACC were cultured in glass coverslips and loaded with fura-2AM by incubation at 25°C in HEPES-buffered salt solution (HSS) containing fura-2AM 2 µmol/L, pluronic F-127 0.2mg/mL and BSA 0.2% w/v for 1 h, protected from light. After loading, coverslips were incubated for an additional hour with HSS containing only BSA to allow intracellular de-esterification of the dye, and then mounted vertically in a continuously perfused (10 mL/min) 2.2 mL cuvette, as described by Byron and Taylor (1993). Temperature of the perfusate was maintained at approximately 22ºC. Fluorescence (excitation 340 and 380 nm, emission 510 nm) was monitored continuously with a Perkin-Elmer LS50B spectrofluorimeter (Perkin-Elmer, Waltham, MA, USA), and treatments were added to the perfusate solution. For $[Ca^{2+}]$ calibration, fluorescence at zero-calcium (R_{min}) and saturated calcium concentrations (R_{max}) were determined. R_{min} was determined by perfusion with Ca^{2+}-free HSS containing ionomycin 1 µmol/L, EGTA 5 mmol/L, KCl 140 mmol/L, and BAPTA 10 µmol/L, and R_{max} by perfusing cells with digitonin 20 µmol/L in complete HSS. Autofluorescence determinations were made at the end of each experiment by perfusing cells with Ca^{2+}-free HSS containing $MgCl_2$ 10 mmol/L and digitonin 20 µmol/L. Fluorescence ratios were calculated after subtraction of autofluorescence and the ratios were converted to $[Ca^{2+}]$ by application of the Grynkiewicz equation (Grynkiewicz *et al.* 1985) with the FL Winlab 3.00 software (Perkin-Elmer). The K_d value appropriate to the temperature and pH was used.

Statistical analysis

Data are expressed as mean + SEM and were analyzed by one-way ANOVA followed by Duncan's *post hoc* test using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at the $p < 0.05$ level.

Results

Effects of retinol on TH phosphorylation and activity

Bovine adrenal chromaffin cells were treated with retinol (10 µmol/L) for up to 2 h, and the phosphorylation of TH at ser40, ser31, and ser19 was determined (Fig. 1a–c). Retinol caused an increase in ser40 and ser31 phosphorylation levels over this time course, but had no effect on ser19 phosphorylation. The increases in phosphorylation of ser40 and ser31 were distinct. Ser40 phosphorylation was increased acutely reaching a maximum (fourfold) at 15–20 min and then remained at an increased level (threefold) up to 2 h (Fig. 1a). Ser31 phosphorylation slowly increased over time reaching a threefold increase at 2 h (Fig. 1b). One possibility for these
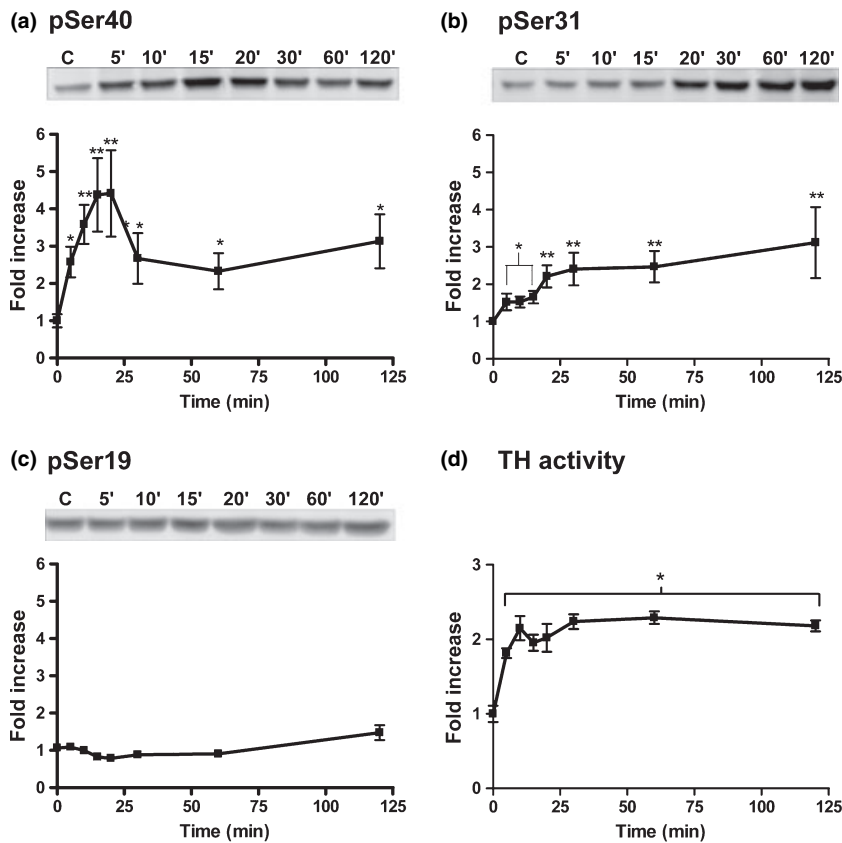
(a) pSer40

(b) pSer31

(c) pSer19

(d) TH activity

Fig. 1 Effects of retinol on TH phosphorylation at ser40, 31, 19 and on TH activity. BACC were incubated with retinol 10 μmol/L for times up to 2 h. Phosphorylation of TH at ser40 (a), ser31 (b), ser19 (c), and TH activity (d) were analyzed and quantified. Values are mean (± SEM) fold increases relative to controls, $N = 9$. Representative gels are shown. *$p < 0.05$; **$p < 0.01$ using one-way ANOVA followed by Duncan's *post hoc* test.

different profiles in ser40 and ser31 phosphorylation was that retinol increased TH phosphorylation by activating distinct signaling pathways in BACC.

Tyrosine hydroxylase activity was increased by retinol after 5 min, reached maximum levels (twofold) at 10 min and then remained at this level up to 2 h (Fig. 1d). This data suggested that if TH phosphorylation caused the increase in TH activity then both ser40 and ser31 were involved.

The effect of different concentrations of retinol on TH phosphorylation was examined. Ser40 phosphorylation was determined at 15 min and ser31 phosphorylation at 2 h, because these were the times at which each site exhibited maximal phosphorylation after retinol treatment (Fig. 1). At 15 min, the effect of retinol on ser40 phosphorylation was first observed at 7 μmol/L and increased up to 20 μmol/L (Fig. 2a). Retinol exposure for 2 h increased ser31 phosphorylation in a concentration-dependent manner beginning at 7 μmol/L and being maximal at 10 μmol/L (Fig. 2b). All the subsequent experiments in this paper were undertaken using 10 μmol/L retinol, as this was near maximal for ser40 phosphorylation and optimal for ser31 phosphorylation. There was no significant effect of retinol on ser19 phosphorylation at any concentration (not shown). Experiments using retinoic acid (1 nmol/L–10 μmol/L) showed that this metabolite of retinol had no significant effect on TH phosphorylation of ser40, or ser31, at any time up to 2 h (not shown).

Effects of protein kinase inhibitors on TH phosphorylation at ser40 and ser31 and on TH activity

The data in Fig. 1 suggested that retinol stimulated TH phosphorylation at ser40 and ser31 via different signal transduction pathways. BACC were therefore incubated with a range of protein kinase inhibitors that have been established to be effective on TH phosphorylation in these cells at the concentrations used here (Dunkley *et al.* 2004). Ser40 phosphorylation was measured at 15 min while ser31 phosphorylation was measured at 2 h. The protein kinase A, p38 and protein kinase inhibitors (H89 10 μmol/L, SB203580 1 μmol/L and KT 5823 300 nmol/L) had no effects on either ser40 or ser31 phosphorylation (data not shown). The PKC inhibitor GÖ6983 (10 μmol/L) completely inhibited ser40 phosphorylation (Fig. 3a), but not ser31 phosphorylation (Fig. 3b). The ERK 1/2 inhibitor UO126 (10 μmol/L) completely inhibited ser31 phosphorylation (Fig. 3b), but not ser40 phosphorylation (Fig. 3a).

We also determined the effect of these inhibitors on TH activity (Fig. 3a and b); GÖ6983 completely inhibited retinol-induced TH activation at 15 min, but had no effect at 2 h, while UO126 had no effect on TH activity at 15 min, but completely inhibited TH activity at 2 h.
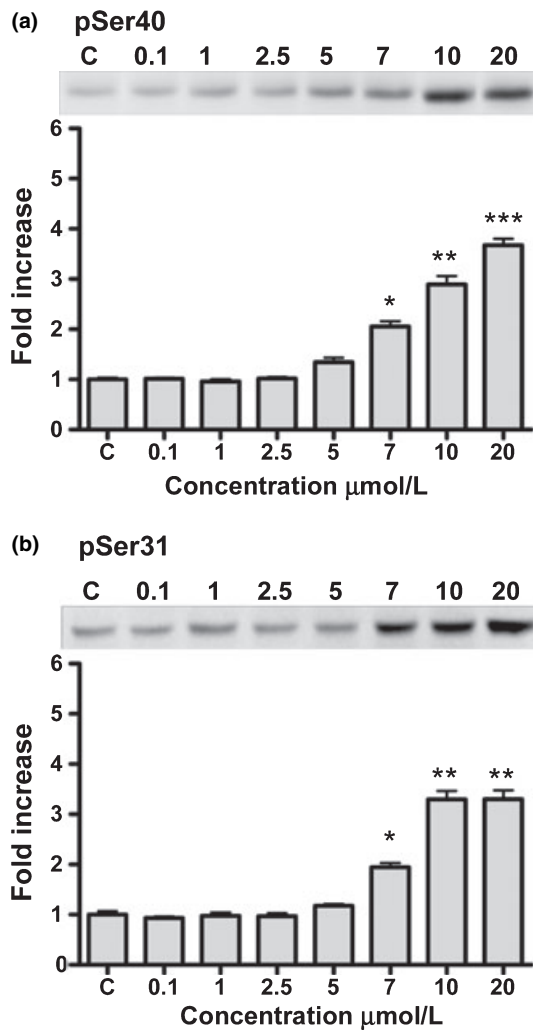
(a)   pSer40

(b)   pSer31

Fig. 2 Effect of retinol concentration on TH phosphorylation at ser40 and ser31. BACC were incubated for 2 h with retinol (0.1, 1, 2.5, 5, 7, 10, and 20 μmol/L). Phosphorylation of TH at ser40 was analysed after 15 min (a) and phosphorylation at ser31 was analyzed after 2 h (b). Values are mean (± SEM) fold increase relative to controls. $N = 9$. Representative gels are shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ using one-way ANOVA followed by Duncan's *post hoc* test.

To confirm the effects of retinol on PKC and ERK 1/2 we assessed the activation of these kinases. Using an antibody that detects PKC substrate phosphorylation, a number of proteins showed increased phosphorylation at 15 min and this effect was blocked by the PKC inhibitor GÖ6983 (Fig. 3c and e). Blots with an antibody against phosphorylated (and activated) ERK 1/2 showed increased phosphorylation at 2 h that was blocked by the ERK 1/2 inhibitor UO126 (Figs. 3d and e). No activation of ERK at 15 min or PKC substrate phosphorylation at 2 h was observed (not shown). These data suggest that retinol increases PKC activity leading to the phosphorylation of a number of proteins, including TH at ser40, and subse-quently increases ERK 1/2 activity leading to TH phosphorylation at ser31. These phosphorylation changes then activate TH.

Effects of reactive oxygen species on TH phosphorylation at ser40 and ser31 and on TH activity

In other cells, retinol activates protein kinases by producing ROS (Gelain *et al.* 2006). We therefore measured cellular ROS production in BACC treated with retinol using the real-time DCFH fluorescent assay (Fig. 4a). Retinol (10 μmol/L) induced a steady increase in cellular ROS production. The increase as a result of 10 μmol/L retinol was abolished by antioxidant pre-treatment with NAC (1 mmol/L).

We then determined the effect of antioxidant pre-treatment with NAC, or DTT (1 mmol/L), on ser40 and ser31 phosphorylation. We observed no effect of NAC, or DTT, on retinol-induced ser40 phosphorylation or TH activation at 15 min (Fig. 4b); however, both antioxidants blocked ser31 phosphorylation and TH activity at 2 h (Fig. 4c). Moreover, PKC activation at 15 min was not blocked by antioxidant treatment, while ERK 1/2 phosphorylation at 2 h was (Fig. 4d). Antioxidants had no effect on TH phosphorylation at ser40 or PKC activity at 2 h (not shown).

These data indicate that retinol treatment leads to ROS production, that ROS has no effect on ser40 phosphorylation or TH activity at 15 min, but leads to an increase in ERK 1/2 activity at 2 h, which increases ser31 phosphorylation and TH activity.

Effects of extracellular calcium on TH phosphorylation at ser40 and ser31 and on TH activity

Protein kinase C can be activated by a variety of stimuli, all of which lead to an increase in intracellular calcium. We therefore examined the effect of the calcium chelator EGTA on retinol-induced ser40 phosphorylation, TH activity and PKC activity at 15 min. EGTA (4 mmol/L) completely blocked the increases in ser40 phosphorylation and TH activity (Fig. 5a), plus PKC activation (Fig. 5c), but had no effect on basal levels. Since EGTA is a non-permeable calcium chelator, these data suggested the involvement of extracellular calcium in the effect of retinol. To confirm this hypothesis, we compared the effect of retinol with nicotine, which is known to depolarize BACC by opening voltage sensitive calcium channels allowing extracellular calcium entry (Marley 2003). Incubation of BACC with nicotine (10 μmol/L) increased TH phosphorylation at ser40 and TH activation (Fig. 5a) and EGTA completely blocked these effects.

We also evaluated the effect of EGTA on TH activation by retinol at 2 h. EGTA inhibited the increases in ser31 phosphorylation and TH activity (Fig. 5b), plus ERK 1/2 activation (Fig. 5c), induced by retinol at 2 h. EGTA also inhibited the increase in cellular ROS production caused by retinol, as seen using the DCFH-DA assay (Fig. 5d).
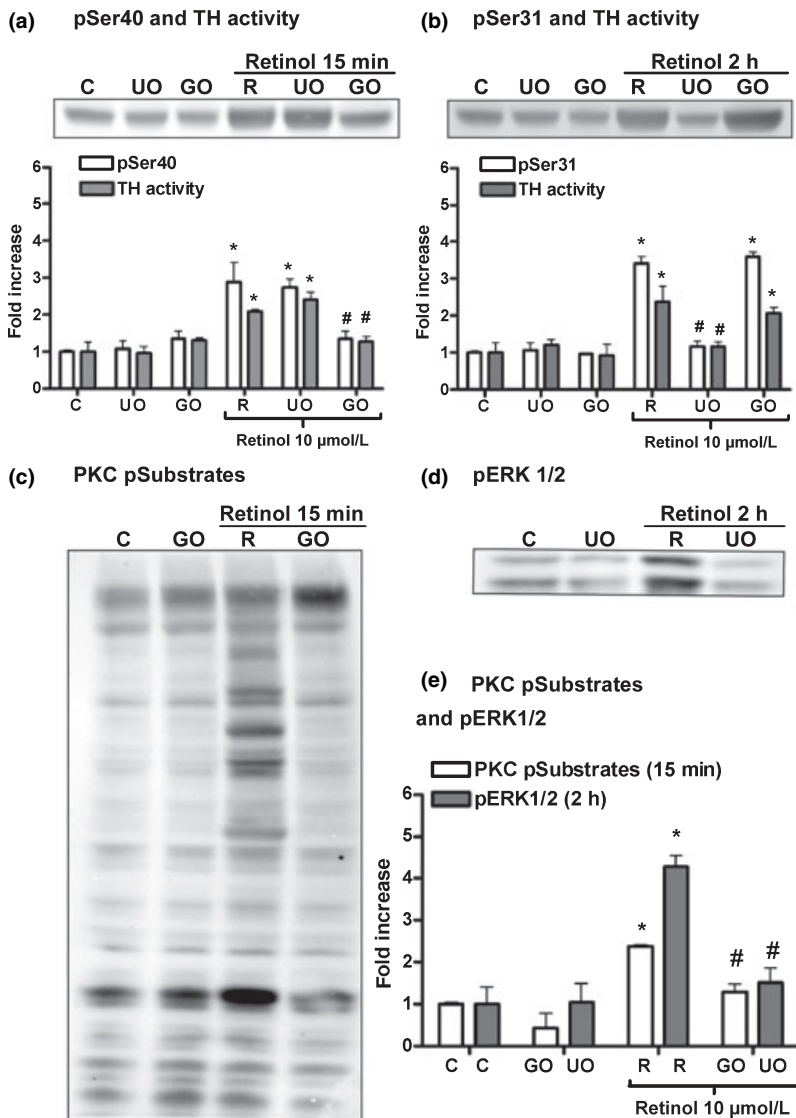
(a) pSer40 and TH activity

(b) pSer31 and TH activity

(c) PKC pSubstrates

(d) pERK 1/2

(e) PKC pSubstrates and pERK1/2

Fig. 3 Effect of protein kinase inhibitors on retinol-stimulated TH phosphorylation and TH activity, and on the activation of PKC and ERK1/2. BACC were pre-incubated for 30 min with GÖ6983 10 μmol/L (GO), or UO126 10 μmol/L (UO) and then incubated with retinol 10 μmol/L (R) for 15 min or 2 h. TH phosphorylation at ser40 and activity at 15 min (a) and TH phosphorylation at ser31 and activity at 2 h (b) were analyzed and quantified. Representative immunoblots for PKC-phosphorylated proteins at 15 min of retinol incubation (c), and the dually phosphorylated ERK 1/2 at 2 h are shown (d). Gels for PKC and ERK 1/2 were analyzed and quantified (e). Values are mean (± SEM) fold increase relative to controls. $N = 9$. *Different from control, $p < 0.05$; #different from retinol treatment, $p < 0.05$; using one-way ANOVA with Duncan's *post hoc* test.

These data suggest that retinol, like nicotine, causes the entry of extracellular calcium into BACC, which directly activates PKC leading to the phosphorylation of TH at ser40 and TH activation within 15 min. The entry of extracellular calcium then induces ROS production and this activates ERK 1/2, ser31 phosphorylation and TH activation at 2 h.

Effects of retinol on extracellular calcium influx

To confirm that retinol caused the entry of extracellular calcium into BACC, we measured the changes in intracellular calcium concentrations ($[Ca_i]$) during retinol treatment using the fura-2AM assay. Addition of retinol caused a gradual increase in $[Ca_i]$ over time (Fig. 6a), which was not observed in the presence of EGTA (Fig. 6b). Similar results to those with EGTA were seen when retinol was added to cells in a calcium-free buffer (not shown). Addition of nicotine caused a rapid increase in $[Ca_i]$ which peaked at 5 min and decreased to a new steady state at 10 min

(Fig. 6c). All of the changes in $[Ca_i]$ induced by nicotine were totally abolished by EGTA treatment (Fig. 6d). Similar results to those with EGTA were seen when nicotine was added to cells in a calcium-free buffer (not shown). Blocking ROS production with NAC or DTT had no effect on the increase in $[Ca_i]$ induced by retinol (Fig. 6e and f).

These results confirm that retinol induces extracellular calcium influx into BACC, but the mechanism differs from that caused by nicotine. They also indicate that ROS production is not required for the initiation or the continued entry of calcium into BACC. The data confirm that ROS production is downstream of retinol-induced calcium entry into BACC.

Discussion

Retinol metabolism can actively influence processes related to motor and cognitive function, both in physiological and
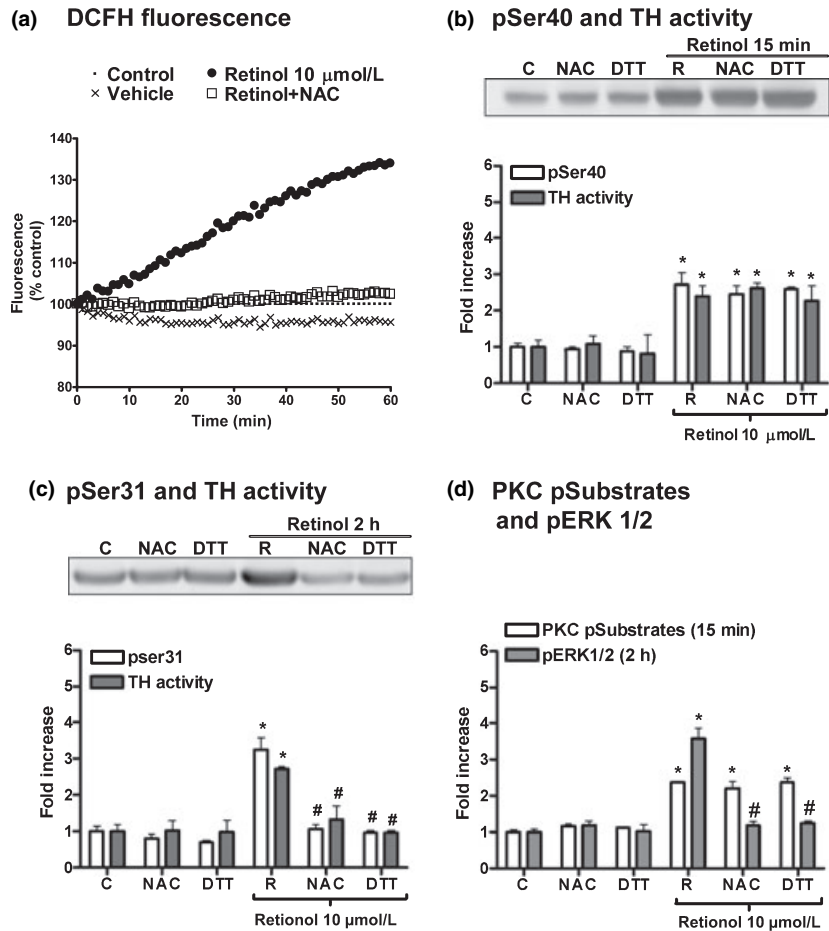
Fig. 4 Effect of reactive oxygen species on TH phosphorylation at ser40, ser31 and on TH activity. BACC were pre-incubated with DCFH-DA 100 μmol/L for 30 min, and the fluorescence caused by ROS formation during incubation with retinol (0–10 μmol/L) was monitored for 60 min; a representative graph of kinetics of ROS formation under these conditions is shown in (a). To evaluate the effect of ROS on TH phosphorylation and activity, BACC were pre-incubated for 30 min with the antioxidants N-acetyl-cysteine 1 mmol/L (NAC) and DTT (1 mmol/L) and then incubated with retinol 10 μmol/L (R) for 15 min or 2 h. TH phosphorylation at ser40 and TH activity at 15 min (b), TH phosphorylation at ser31 and TH activity at 2 h (c), PKC activation and ERK 1/2 phosphorylation (d) were analysed and quantified. H_2O_2 was used as a positive control for ROS production (not shown). Values are mean (± SEM) fold increase relative to controls. $N = 9$. Representative gels are shown. *Different from control, $p < 0.05$; #Different from retinol treatment, $p < 0.05$; using one-way ANOVA with Duncan's post hoc test.

pathological conditions. Chronic vitamin A deprivation was reported to cause motor dysfunction in rats, but this was not the result of an effect on dopamine neurons (Carta et al. 2006). Acute models of vitamin A administration in cell cultures, however, show different results. RA, considered the most biologically active metabolite of retinol, is recognized as one of the main regulators of nervous system development, and it is widely used as an inducer of in vitro neural differentiation in selected cell lines (Pan et al. 2005). RA receptor and/or RX receptor-mediated gene transcription plays a role in these processes, modulating TH activity by increasing its expression (Cosgaya et al. 1996; Patel et al. 2006). TH expression could be directly up-regulated by RA receptor binding at the promoter region of TH gene (Jeong et al. 2006). We did not observe any effect on TH phosphorylation induced by RA suggesting that the effects seen here were not regulated by RA receptor. Recent work has found that retinoids exert significant non-genomic (also called extra-nuclear) actions on neurons and the TH-expressing cell line PC12, by mechanisms that influence signaling pathways (Canon et al. 2004). These effects were switched on within an hour and lasted many days. However, effects of retinoids on TH regulation have not previously

been investigated. In this study, we demonstrated for the first time that through Ca^{2+} influx retinol induced the phosphorylation of TH at ser40 and ser31 by two distinct mechanisms, each leading to an increase in enzyme activity. These sequential effects of retinol were both compatible with non-genomic actions, since they were detectable at such early times.

Other stimuli that are physiologically relevant to BACC have been observed to induce quite different profiles of TH phosphorylation over time when compared with the results obtained here (Bunn et al. 1995; Bobrovskaya et al. 2001, 2007a,b; Dunkley et al. 2004). The major differences are that retinol does not induce Ser19 phosphorylation and the retinol-induced increases in Ser40 and Ser31 phosphorylation are substantially delayed. These differences between the phosphorylation time courses induced by retinol and other stimulators of BACC are most likely related to the fact that the other stimulators all act via receptors and ion channels leading to very rapid and substantial changes in intracellular second messengers, such as calcium and cyclic AMP. There is no evidence to date of membrane receptors for retinol. Retinol is a lipophilic molecule that can interact with cell membranes directly, producing changes in membrane fluidity
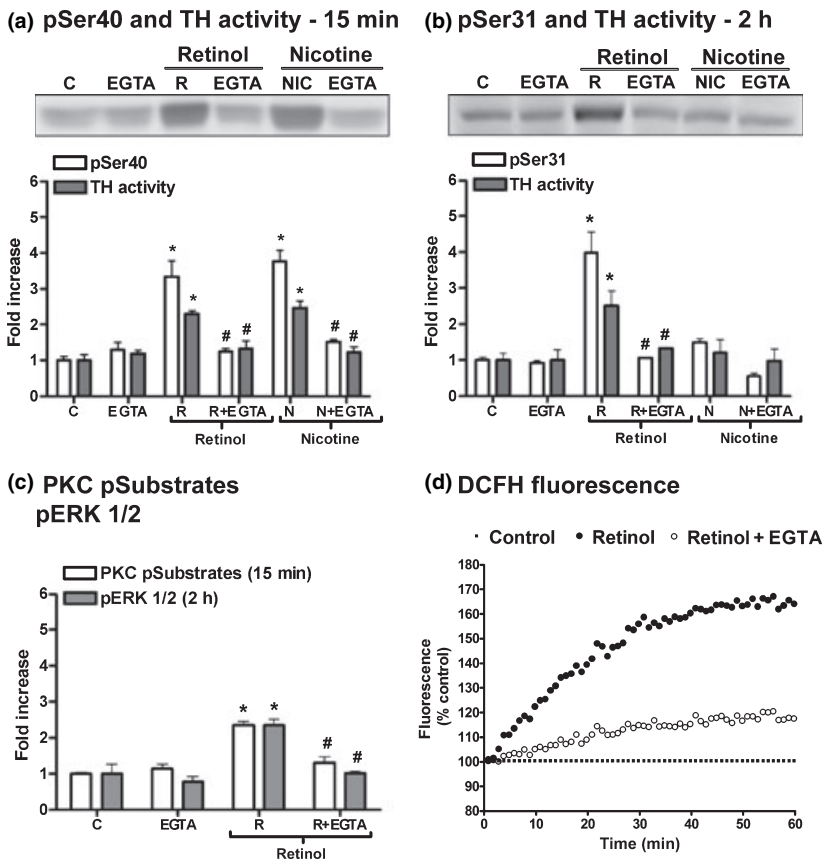
(a) pSer40 and TH activity - 15 min

(b) pSer31 and TH activity - 2 h

(c) PKC pSubstrates pERK 1/2

(d) DCFH fluorescence

Fig. 5 Effects of extracellular calcium on TH phosphorylation and TH activity. BACC were pre-incubated with EGTA 4 mmol/L for 30 min, and then incubated with either retinol 10 μmol/L (R) or nicotine 10 μmol/L (N) for 15 min or 2 h. TH phosphorylation at ser40 and TH activity at 15 min (a), TH phosphorylation at ser31 and TH activity at 2 h (b), PKC and ERK 1/2 activation (c) were analyzed and quantified. Kinetics of ROS production by retinol in the presence of EGTA was analyzed by DCFH assay; a representative graph is shown in (d). Values are mean ($\pm$ SEM) fold increase relative to controls. $N = 9$. *Different from control, $p < 0.05$; #Different from retinol treatment, $p < 0.05$; using one-way ANOVA with Duncan's *post hoc* test.

and membrane potential (Loss *et al.* 1998; Kim *et al.*, 1988). This takes some time as the data on intracellular calcium showed only a slow and steady rise in intracellular calcium in response to retinol. This was in contrast to nicotine which showed a very large and extremely rapid initial increase in intracellular calcium. The reason that ser19 was not phosphorylated and ser40 and ser31 phosphorylation was delayed with retinol, is therefore most likely because the very slow and steady increase in intracellular calcium failed to substantially activate a range of protein kinases that were activated by agents such as nicotine.

At least eight different kinases can phosphorylate TH at ser40 in BACC (Dunkley *et al.* 2004). We found that PKC inhibitor GÖ6983 completely inhibited the effects of retinol on ser40. We also confirmed that PKC was activated by retinol and inhibited by GÖ6983. The calcium-dependent α subunit of PKC is present in BACC (Dunkley *et al.* 2004). The rapid rise in calcium induced by nicotine induces the activation of a range of protein kinases that can all phosphorylate ser40 (Dunkley *et al.* 2004). These then become inactivated over time, except for PKC whose activity is sustained for days (Bobrovskaya *et al.* 2007a). A much slower rise in intracellular calcium with retinol would not increase all of the protein kinases activated by nicotine, but it activated PKC.

Although the effects of retinol and nicotine on intracellular calcium were both dependent on extracellular calcium, there were substantial differences in the responses evoked by each stimulus. Nicotine effects are mediated by nicotinic receptor activation, which induces membrane depolarization and consequent opening of voltage-operated calcium channels causing the acute rise in intracellular calcium. This was followed by a fall in calcium levels and then a sustained phase of increased calcium, which is the result of the entry of calcium through store-operated calcium channels (Marley 2003). The initial increase in calcium induced by retinol was much slower and there was no peak and sustained phase, suggesting that there was no contribution from intracellular calcium. Our experiments with fura-2AM also suggested that the effects of retinol were entirely dependent on extracellular calcium and not on intracellular calcium as; (i) EGTA completely inhibited the effects of retinol on TH phosphorylation, (ii) incubation without extracellular calcium produced the same results as with EGTA; (iii) agents known to increase intracellular calcium by mobilizing intracellular stores, such as caffeine, were not affected by EGTA or calcium-free medium (Gelain *et al.*, unpublished data).

Ser31 phosphorylation induced by retinol was dependent on ERK 1/2 activation. ERK 1/2 is activated in BACC by both PKC-dependent and PKC-independent pathways
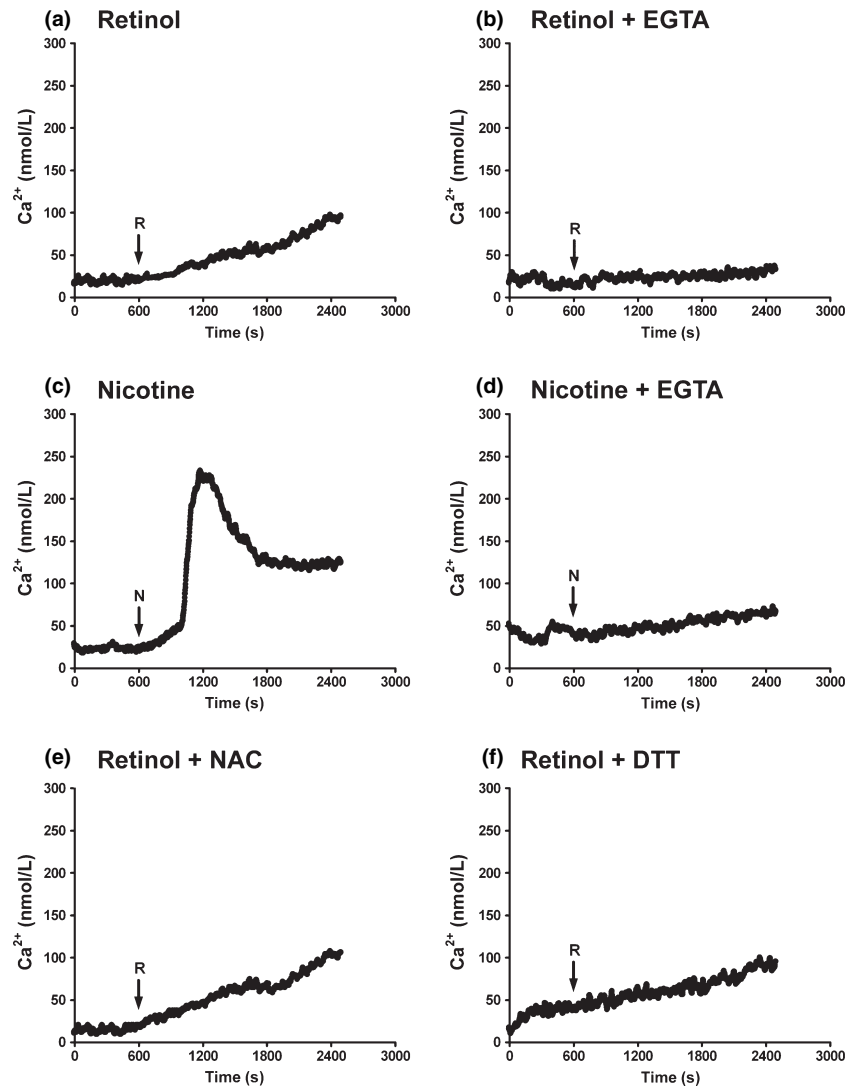
(a) Retinol

(b) Retinol + EGTA

(c) Nicotine

(d) Nicotine + EGTA

Fig. 6 Changes in cytosolic calcium induced by retinol and nicotine. Cultured cells attached to glass coverslips loaded with fura-2AM were attached to a perfusion system containing calcium, and the fluorescence was constantly monitored. Retinol 10 µmol/L (a) or nicotine 10 µmol/L (b) were added to the perfusion medium when indicated by the arrows. The effect of retinol or nicotine addition to BACC perfused with medium containing EGTA 4 mmol/L (b and d), and the effect of retinol addition to BACC perfused with medium containing NAC 1 mmol/L (e) or DTT 1 mmol/L (f) were also evaluated. Representative traces from three different experiments are shown.

(e) Retinol + NAC

(f) Retinol + DTT

(Dunkley *et al.* 2004). We found no evidence for PKC involvement since GÖ6983 had no effect on ERK 1/2 or ser31 phosphorylation. We demonstrated that the increase in calcium induced by retinol was directly correlated to the increase in cellular ROS production. ROS induction by retinol has been observed *in vitro* and *in vivo*, but the mechanism of this phenomenon is not known (Murata and Kawanishi 2000; Dal-Pizzol *et al.* 2001; Klamt *et al.* 2005). ERK 1/2 activation and Ser31 phosphorylation were inhibited by antioxidant treatment with DTT and NAC, confirming the involvement of ROS in the effects of retinol on ERK 1/2. Retinol has previously been shown to activate ERK 1/2 and related pathways by a ROS-dependent mechanism (Gelain *et al.* 2006). It was previously shown that calcium may be involved in the genesis of ROS production in other cells (Ha and Park 2006; Schild and Reiser 2005; Zhang *et al.*, 2006b), but this is the first time the calcium was observed to be involved in retinol-induced ROS.

Tyrosine hydroxylase activation can be mediated by phosphorylation of ser40 or ser31 (Dunkley *et al.* 2004). Under resting conditions, TH is inhibited by the almost irreversible binding of catechols to its catalytic site. Phosphorylation of TH at ser40 changes its conformation and makes catecholamine binding readily reversible, thereby activating TH (Ramsey and Fitzpatrick 1998). In this study, we found that TH activation by retinol treatment occurred as a result of the phosphorylation of ser40 at 15 min. We also found that TH activation by retinol treatment occurred because of the phosphorylation of ser31 and not ser40 at 2 h. The mechanism for this increase in TH activity is most likely because of a change in the affinity of the enzyme for its cofactor BH4 (Dunkley *et al.* 2004). These increases in TH activity, because of ser40 or ser31 phosphorylation, would lead to increases in catecholamine synthesis (Dunkley *et al.* 2004), and therefore be of physiological importance at different times after exposure of the chromaffin cells to retinol.

Tyrosine hydroxylase activation is the primary mechanism for replenishment of secreted catecholamines in adrenal medullary cells. Physiological receptor agonists compensate for the loss of secreted catecholamines by activating TH in the short-term (by phosphorylation) and in the long-term (by increasing protein expression). What is the role of retinol-induced TH activation *in vivo*? Serum levels of retinol in healthy subjects varies between 2–3 μmol/L (Boonsiri *et al.* 2007), and these levels are primarily controlled by diet. Fast, non-genomic actions of retinol and its derivatives have therefore been neglected as they do not occur at such low concentrations of retinol. However, in recent years, retinoids have been extensively used in vitamin supplements and tested as therapeutic agents for malignant (Abu *et al.* 2005) and neurodegenerative diseases (Ono and Yamada 2007). Supplementation protocols have been tested in both humans and animals utilizing different forms of administration, such as intravenous and intramuscular injections, as well as oral administration (Macapinlac and Olson 1981; Adamson *et al.* 1995; Basu *et al.* 2003), substantially increasing the overall levels of circulating and stored vitamin A (for review, see Ross 1993; and Penniston and Tanumihardjo 2006). It is therefore likely that the effects that we have seen in primary BACC also occur *in vivo*, depending on the dose and mode of administration of the retinoids. This would mean that increases in intracellular calcium could lead to activation of signal transduction pathways and physiological consequences in the adrenal gland.

We have shown that retinol can act to increase intracellular calcium by the entry of extracellular calcium. The rise in intracellular calcium then leads to two distinct and sequential responses one dependent on PKC and the other dependent on ROS and ERK1/2. Both these mechanisms have physiological consequences in respect to TH activation. Similar action of retinol in other cells, leading to PKC activation and ROS production, could have a range of physiological consequences depending on the functions of the cells involved.

Acknowledgments

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Parte 3

Discussão

Desde a observação de Wolbach e Howe, em 1925, de que ratos com deficiência de vitamina A apresentavam acentuada atrofia do baço e do timo, e de sua subseqüente observação de que a administração de ácido retinóico não revertia completamente o quadro de imunodeficiência causada pela hipovitaminose A (Wolbach & Howe, 1978), ficou evidente de que a ação da vitamina A em sistemas biológicos não era exclusivamente causada pela geração de ácido retinóico no organismo. No entanto, a recente caracterização funcional e molecular dos receptores retinóides, bem como dos seus mecanismos de ação transcricional, demonstrou que, além do ácido retinóico, havia apenas mais um composto retinóide capaz de atuar como agonista destes receptores – o ácido retinóico 9-*cis* (Bastien & Rochette-Egly, 2003). Como este isômero é gerado diretamente a partir do ácido retinóico todo-*trans*, a possibilidade de ações independentes da ativação de receptores retinóides se tornou uma hipótese plausível para explicar parte dos efeitos biológicos da vitamina A.

Na tentativa de elucidar esta questão, alguns trabalhos mostraram que certas modificações fisiológicas decorrentes de modificações do estado redox estavam associadas a alterações no status de vitamina A no organismo (Diplock, 1991). Os primeiros trabalhos indicavam um papel antioxidante da vitamina A, mas estudos posteriores demonstraram que a suplementação alimentar de vitamina A aumentava a

incidência de câncer de pulmão e problemas cardiovasculares em populações sujeitas a certos fatores de risco (por exemplo, fumantes e trabalhadores de minas de asbesto – Omenn et al., 1994). Foi inferido, à época, que uma possível ação pró-oxidante poderia explicar os resultados observados, e trabalhos posteriores demonstraram um significativo potencial pró-oxidante da vitamina A em diferentes sistemas, tais como culturas de células, ensaios *in vitro* e em experimentos com animais (Dal-Pizzol et al., 2001; Klamt et al., 2003; Olson 1996). Assim como outras moléculas de reconhecida atividade antioxidante, a vitamina A já é reconhecida como uma molécula redox ativa, protegendo contra a ação de espécies reativas em algumas situações, e promovendo o dano oxidativo em outras. Atualmente, acredita-se que muitos dos efeitos tóxicos observados em casos de hipervitaminose A devam-se a este potencial redox-ativo da vitamina A (Penniston & Tanumihardjo 2006)

No entanto, não são bem conhecidos os contextos exatos nos quais a vitamina A começa a exercer um papel pró-oxidante. Elevações na concentração de retinol estariam entre os fatores determinantes, como observado em trabalhos prévios (Klamt et al., 2003; Dal-Pizzol et al., 2001), mas ainda não são claros os motivos que levam a perturbações no controle da homeostase do metabolismo da vitamina A (Penniston & Tanumihardjo 2006). Além disso, perturbações na homeostase de outras moléculas redox-ativas também podem afetar a atividade redox da vitamina A, como é o caso do ferro (Dal-Pizzol, 2000). No presente trabalho, nós confirmamos que o aumento na produção de espécies reativas intracelulares por retinol, em células vivas, é revertido pela presença de um quelante de ferro (fenantrolina, fig. 2, cap.I), e observamos, pela primeira vez, que um quelante impermeável de cálcio (EGTA), também reverte este

efeito do retinol. Este dado sugere, pela primeira vez, que o cálcio pode estar envolvido no efeito pró-oxidante da vitamina A observado em diversos trabalhos. Não observamos, no entanto, nenhum efeito com concentrações variadas de ácido retinóico, dado este que indica que as ações da vitamina A que não são mimetizadas pelo ácido retinóico podem estar associadas a um mecanismo de ação redox-dependente.

As tentativas de elucidar os mecanismos não-genômicos de sinalização dos retinóides são relativamente bem recentes, com os primeiros trabalhos tratando exclusivamente do assunto datando a partir de 2000. Dos trabalhos disponíveis, há um consenso de que a modulação de proteínas-chave em rotas citoplasmáticas de sinalização é o evento central da ação não-genômica da vitamina A. Entretanto, existe uma discussão sobre o envolvimento ou não dos receptores nucleares retinóides, em nível citoplasmático, nesta modulação. Enquanto alguns trabalhos demonstram que cinases regulatórias, tais como ERK1/2 e PKC, podem ser ativadas em células com receptores retinóides silenciados (Aggarwal et al., 2006), e também que a ligação do retinol a domínios específicos da PKC é capaz de modular a atividade desta cinase (Hoyos et al., 2000), outros trabalhos vêm demonstrando evidências cada vez mais robustas de que receptores retinóides, uma vez ativados, atuam como co-reguladores destas cinases e de outras proteínas regulatórias, exercendo uma função não transcricional, além de seu papel clássico (Cañon et al., 2004; Dey et al., 2007; Masiá et al., 2007). Mas nenhum destes trabalhos explorou, até o momento, a possibilidade de envolvimento de espécies reativas no mecanismo de ação não-genômica da vitamina A.

Aqui, nós observamos uma ativação redox-dependente de ERK1/2 durante o tratamento com retinol nos dois modelos celulares estudados. No entanto, houve uma sensível diferença no tempo de ativação máximo observado, que foi de 15 minutos em células de Sertoli (Fig. 1 cap. II) e de 2 horas em células cromafins (Fig. 3 cap. III). Esta diferença pode ser explicada pela diferença no perfil temporal de produção de espécies reativas, observadas pelo ensaio de fluorescência para espécies reativas intracelulares, ambos correspondendo ao curso de tempo de ativação de ERK1/2 em seus respectivos tipos celulares.

A ativação de ERK1/2 também apresentou um perfil diferente de inibição por antioxidantes nos dois tipos celulares, que provavelmente deve-se às diferenças nos modos de administração destes tratamentos. Em células de Sertoli, os compostos antioxidantes/redutores de cisteína N-Acetilcisteína (NAC) e ditiotreitol (DTT) não reverteram o efeito do retinol (Fig. 2 Cap. II), apesar dos antioxidantes Trolox e manitol terem revertido este efeito, enquanto que nas células cromafins tanto NAC quanto DTT exerceram um claro efeito inibitório (Fig. 4 Cap. III). No entanto, NAC e DTT foram administrados juntamente com o retinol nos experimentos com células de Sertoli, enquanto que nos experimentos com células cromafins estes compostos foram adicionados 30 minutos antes do início do tratamento. Assim, é provável que este tempo de pré-incubação permitiu uma captação mais eficiente destes compostos pelas células cromafins em cultura, ao contrário do que acontece no protocolo de co-incubação, quando os antioxidantes são adicionados às células juntamente com o retinol.

As ERK1/2 são MAPKs classicamente descritas como cinases ativadoras de ciclo celular, controlando cascatas de fosforilação cuja ativação é geralmente associada à ativação de proteínas tais como cdk's e ciclinas. Além disso, também se sabe que as ERK1/2 são ativadoras de um grupo de cinases nucleares chamadas RSKs, que estão envolvidas no controle do estado de fosforilação de histonas (Kim & Lee, 2007). Em trabalhos anteriores, foi observado que o retinol era capaz de aumentar o estado de fosforilação da histona H3, o que é considerado um marcador de ativação de ciclo celular, de maneira redox-dependente (Moreira et al., 2000). É possível que, neste caso, este processo seja mediado, pelo menos em parte, pela modulação redox do estado de ativação das ERK1/2, que já foram descritas como sendo passíveis de modulação por espécies reativas (McCubrey et al., 2006).

É bem estabelecido que pulsos pró-oxidantes servem como estímulo para divisão celular, através da ativação de rotas de sinalização e de fatores de transcrição redox-dependentes. Foi demonstrado que o potencial oncogênico de diversos compostos pró-oxidantes está associado intimamente à superativação destas rotas (McCubrey et al., 2006). Por outro lado, viu-se também que diversos compostos de reconhecida atividade pró-neoplásica também possuíam características pró-oxidantes associadas ao seu efeito (McCubrey et al., 2006). A vitamina A é um reconhecido regulador de processos associados ao ciclo celular, como diferenciação e apoptose (Chambon, 1996). É plausível que parte destes processos sejam regulados de maneira não-genômica e redox-dependente. A transformação pré-neoplásica e a indução de apoptose por retinol já foram observadas em células de Sertoli, ocorrendo

de maneira redox-dependente (Klamt et al., 2003; Klamt et al., 2007). É provável que rotas controladas por ERK1/2 também sejam ativadas nessas situações.

Em células catecolaminérgicas, é bem descrito que as ERK1/2 são as únicas cinases responsáveis pela fosforilação da serina 31 da tirosina hidroxilase, sendo que esaa fosforilação também foi observada em um sistema *in vitro* contendo apenas as duas enzimas (Dunkley et al., 2004). Apesar de inicialmente as ERK1/2 terem sido associadas principalmente a processos associados à regulação do ciclo celular, assim como outras MAPKs, hoje em dia diversos trabalhos mostram o envolvimento destas cinases no controle de outros fenômenos celulares, tais como regulação enzimática pós-transcricional e processos inflamatórios (Yan et al., 2007). No sistema nervoso central, a ativação das ERK1/2 é associada à modulação da plasticidade neural através da ativação do fator de transcrição CREB (Sweatt, 2001). A ativação de enzimas envolvidas na produção de neurotransmissores, como é o caso da tirosina hidroxilase, é outra de suas funções recentemente descritas (Dunkley et al., 2004). A diferenciação *in vitro* de linhagens de células-tronco e neuroblastomas em neurônios especializados têm sido freqüentemente proposta como terapia para doenças neurodegenerativas, com resultados promissores (Sasai, 2002). No caso das doenças de Parkinson e de Huntington, a diferenciação dopaminérgica de neuroblastomas *in vitro* para posterior transplante é realizada através de tratamento prolongado com ácido retinóico, sendo que a imunomarcação de tirosina hidroxilase é considerada o marcador clássico desse tipo de diferenciação (Sasai, 2002), mas os mecanismos intracelulares envolvidos nesse processo não são bem compreendidos. Apenas recentemente o envolvimento de MAPKs vem sendo implicado na diferenciação

dopaminérgica, além da GABAérgica, *in vitro* (Sweatt, 2001), e é provável que exista um forte componente não-genômico, redox-dependente, nesse processo, já que demonstramos que o retinol pode afetar a atividade da tirosina hidroxilase de maneira aguda através da modulação da fosforilação da serina 31 via ERK1/2 e também da serina 40 via PKC (cap. III).

Já havia sido descrito anteriormente que o retinol era capaz de modular a atividade de PKC através de uma interação molecular direta, como mencionado anteriormente (Hoyos et al., 2000); mas essa interação, por si só, não aumentava a atividade da PKC (apesar de modificar sua conformação), o que só acontecia na presença de um estímulo pró-oxidante, no caso a co-incubação com peróxido de hidrogênio. Este dado também reforça a hipótese de que o retinol é capaz de exercer um efeito sinalizador independentemente do ácido retinóico e dos receptores retinóides, e de que esse efeito envolve um mecanismo redox-dependente. No entanto, para nossa surpresa, a ativação de PKC pelo retinol observada nas células cromafins não foi revertida pelo pré-tratamento com NAC e DTT (Fig. 4, cap. III). Experimentos subseqüentes demonstraram que o retinol causava um aumento gradual de cálcio intracelular durante o período de incubação, e que este aumento devia-se a um influxo de cálcio extracelular (Fig. 6 cap. III). Além disso, observamos que a ativação redox-dependente de ERK1/2 nessas células (e conseqüente sustentação da ativação da tirosina hidroxilase pela fosforilação da serina 31) também era revertida pela pré-incubação com EGTA, confirmando os dados observados com as células PC12 (Fig. 1-2, cap.I).

Esses resultados estão de acordo com a idéia mais ou menos generalizada na literatura de que o potencial antioxidante ou pró-oxidante da vitamina A é altamente dependente do perfil redox do micro-ambiente onde ela se encontra, o qual por sua vez é contextualizado por diversas outras moléculas redox-ativas. Apesar de ser claro, pelos nossos resultados, que o cálcio é essencial no processo de geração de espécies reativas intracelulares pela vitamina A, não temos como afirmar ainda qual o seu papel exato nesse fenômeno, nem por qual mecanismo isto acontece. No entanto, esse dado abre novas perspectivas no estudo dos mecanismos de sinalização não-genômica da vitamina A, uma vez que o envolvimento desse importante sinalizador intracelular não tem sido considerado nesse efeito até o presente momento. É possível, por exemplo, que as diferenças entre a produção de espécies reativas intracelulares induzidas por retinol, em diferentes tipos celulares, sejam devidas a diferentes papéis exercidos pelo cálcio nessas células, e a presença de diferentes tipos canais de cálcio nas membranas dessas células.

Diversas evidências, diretas e indiretas, já sugeriam que a sinalização não-genômica por vitamina A poderia envolver um mecanismo redox-dependente. Muitas das proteínas componentes de rotas centrais de sinalização celular possuem reconhecidos domínios regulatórios redox-sensíveis. Neste sentido, domínios contendo resíduos de cisteína são particularmente passíveis deste tipo de regulação. Certos fatores de transcrição têm sua atividade aumentada por espécies reativas através da modificação do estado de oxidação dos resíduos cisteína, como AP-1 e NF-κB (Sun & Oberley, 1996). As rotas reguladas pelas principais MAPKs – ERK1/2,

BMK1, JNK e p38 – também são reconhecidamente redox-sensíveis, e exercem um papel central na resposta celular a insultos oxidativos (McCubrey et al., 2006). Todas as PKCs consistem de um domínio N-terminal regulatório e um domínio C-terminal catalítico, e na forma inativa da PKC esses domínios se mantêm ligados por interações intramoleculares; um dos modelos propostos para modulação da atividade da PKC é a oxidação de domínios ricos em cisteína localizados entre esses dois domínios, o que facilitaria a conversão à forma ativa, já que as PKCs são reconhecidamente sensíveis a espécies reativas (Taniguchi et al., 2006). A ativação de PKC também foi observada com ácido retinóico, e como esse composto é originado intracelularmente a partir do retinol, é possível que ambos modulem a atividade dessa cinase concomitantemente.

A ativação de ERK1/2 foi observada também com ácido retinóico em outros modelos celulares e nestes casos ocorre tanto em células super-expressando receptores retinóides (Cañon et al., 2004) quanto em linhagens com receptores silenciados (Aggarwal et al., 2006). Nós observamos que a incubação com diferentes concentrações de ácido retinóico em células PC12, ao contrário do retinol, não causou qualquer efeito sobre a produção de fluorescência dependente de espécies reativas, pelo ensaio de DCFH em tempo real (Fig. 1, Cap. I). As células PC12 foram as mesmas utilizadas por Cañon et al. para demonstrar que o ácido retinóico é capaz de ativar ERK1/2 e CREB por uma via não-genômica, e nós constatamos que as mesmas concentrações utilizadas pelo grupo espanhol não produziram nenhum efeito pró-oxidante nessas células. Este dado sugere que o mecanismo de ativação de rotas não-genômicas pelo ácido retinóico possa apresentar diferenças com o mecanismo

pelo qual este fenômeno acontece na presença do retinol, uma possibilidade até agora não cogitada na literatura. Isto explicaria algumas diferenças observadas entre os efeitos não-genômicos do retinol e do ácido retinóico, sendo possível que o controle do metabolismo intracelular do retinol poderia modular as respostas não-genômicas destes dois compostos. Além disso, esse controle possibilitaria uma mudança de sinalização não-genômica (dependente de retinol) para genômica (dependente exclusivamente de ácido retinóico, via RAR e RXR), de acordo com as necessidades da célula.

Concluindo, baseados nos dados apresentados nos três capítulos desta tese, nós propomos que o mecanismo de sinalização não-genômica do retinol envolve a produção de espécies reativas em nível celular, sendo essas espécies responsáveis pela ativação de rotas de sinalização citoplasmáticas de maneira independente de receptores retinóides. As peculiaridades desse mecanismo ainda necessitam de mais estudos para serem compreendidas, mas certamente envolvem a participação de cálcio extracelular, ferro e produção de superóxido mitocondrial. A ordem seqüencial dos eventos celulares apresentados nesta tese encontra-se sumarizada nos diagramas apresentados respectivamente nas figuras 2 e 3, nas duas próximas páginas.
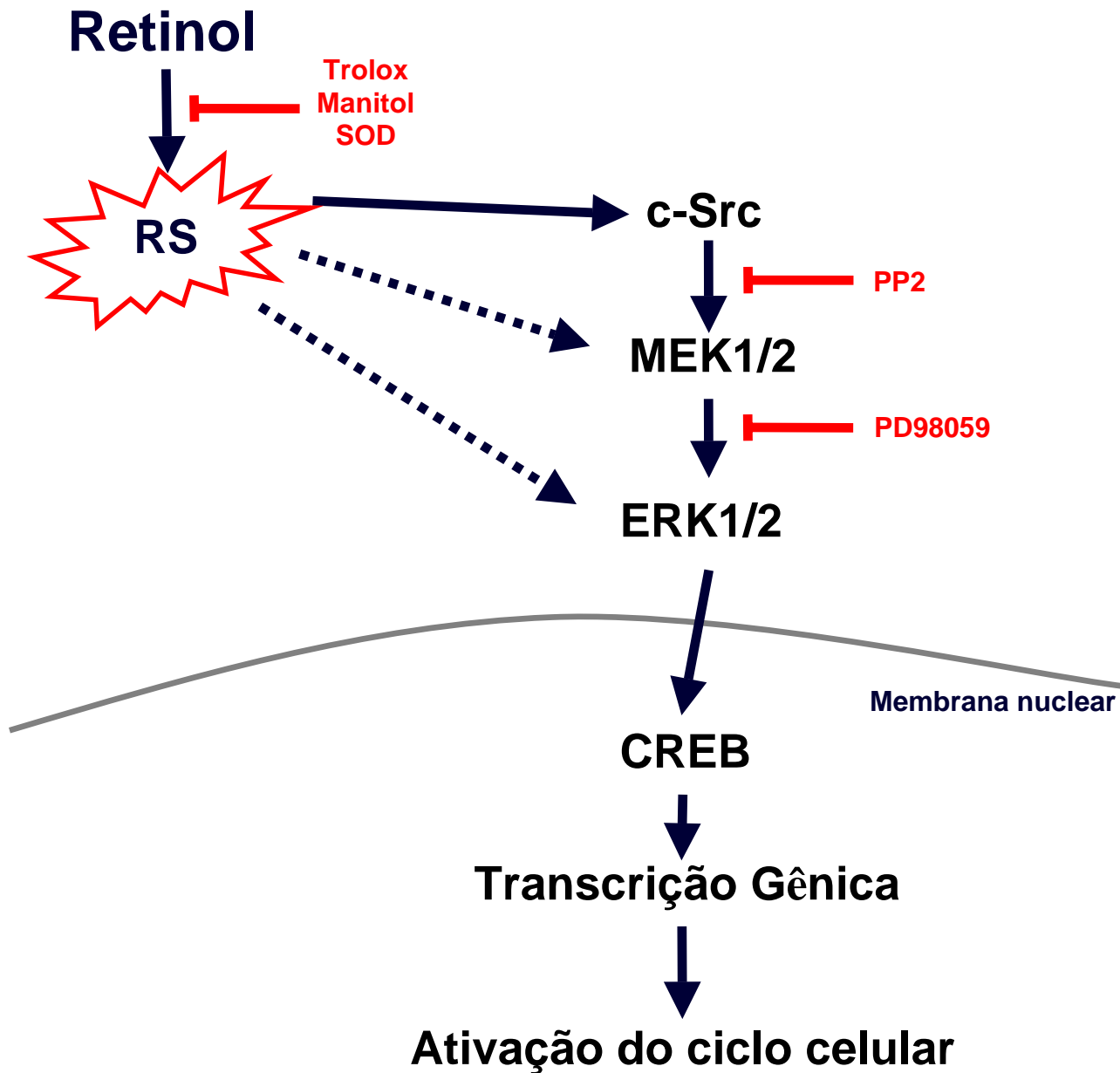
Fig.2 Ativação redox-dependente da rota c-Src/MEK/ERK/CREB em células de Sertoli por retinol.
A incubação com retinol 7 µM causa uma aumento na produção de espécies reativas (RS), que é revertido na presença de Trolox, manitol ou superóxido dismutase (SOD). Este aumento induz uma cascata de fosforilação seqüencial da s-Src tirosina cinase, MEK1/2 e ERK1/2, e a seqüência de ativação dessas cinases foi determinada pelo uso de inibidores farmacológicos específicos (PP2 e PD98059). A ativação dessa rota de sinalização causa um aumento na fosforilação do fator de transcrição CREB, levando a uma aumento na sua atividade regulatória na região promotora de diversos genes.
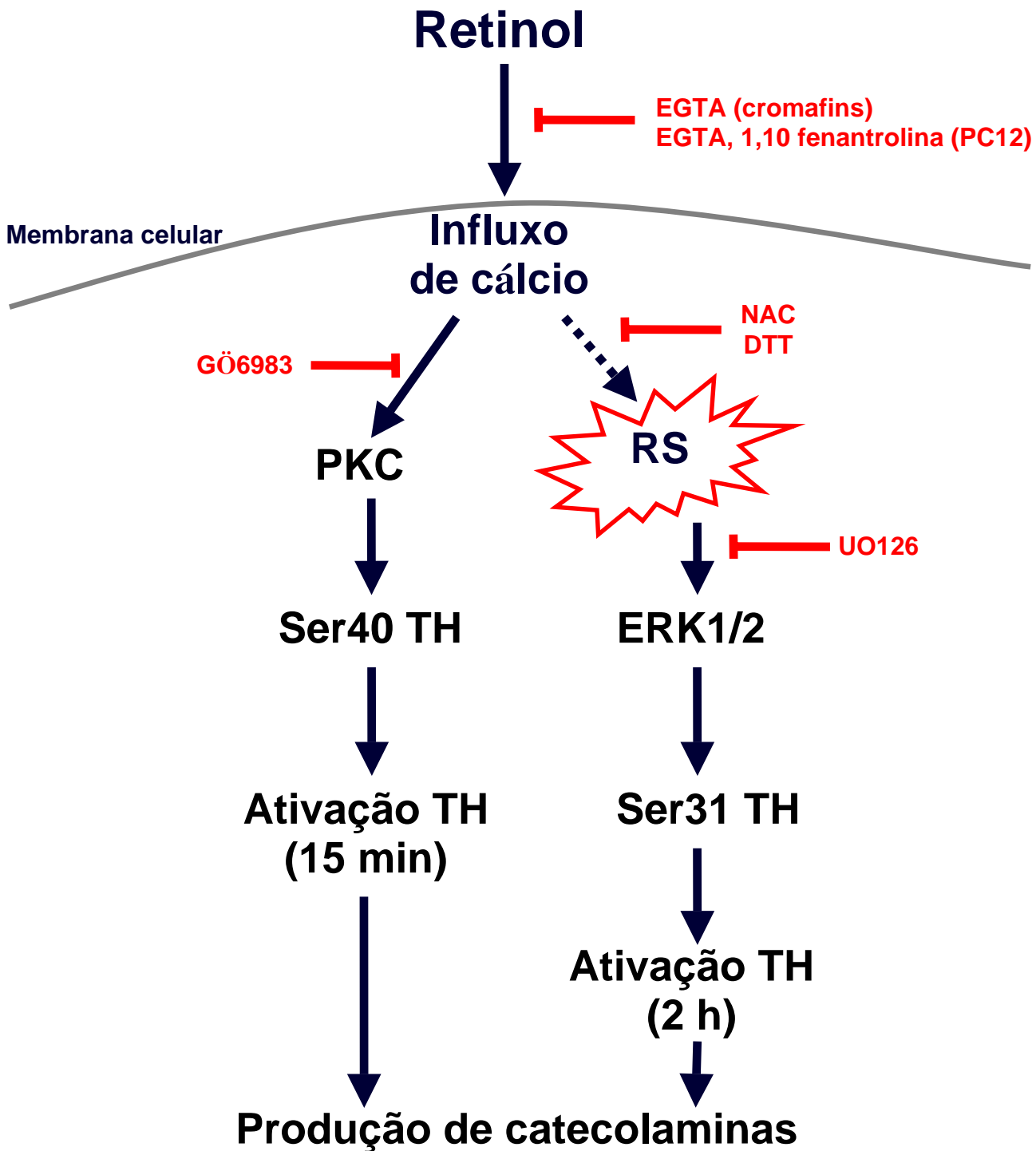
Fig.3 Ativação da tirosina hidroxilase em células cromafins por retinol 10 μM. O retinol induz um aumento no influxo celular de cálcio, efeito que é inibido por EGTA. Este primeiro efeito induz a ativação de PKC em 15 minutos e conseqüente fosforilação da TH na serina 40 (Ser40 TH) e ativação enzimática, efeito inibido pelo inibidor de PKC GÖ6983. Além disso, o retinol ativa a produção de espécies reativas (RS), a qual aumenta gradativamente ao longo de duas horas, resultando na ativação de ERK1/2 e conseqüente fosforilação da TH na serina 31 (Ser31 TH), sustentando a ativação enzimática após duas horas de incubação. A produção de RS é dependente do cálcio, e é bloqueada pelos antioxidantes N-acetilcisteína (NAC) e ditiotreitol (DTT). A produção de RS em células PC12 também é inibida pelo quelante de cálcio EGTA e pelo quelante de ferro 1,10-fenantrolina.

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Anexos:

-Relatório de Estágio no Exterior (PDEE-CAPES)

-Parecer do Professor Orientador no Exterior

-Young Investigator Award 2007: South American Group of the Society for Free Radical Biology and Medicine

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA

Efeito da vitamina A (retinol) na regulação da enzima Tirosina Hidroxilase

Relatório referente ao período de estágio no exterior (University of Newcastle, Australia) financiado pela bolsa do Programa de Doutorado no País com Estágio no Exterior (PDEE-CAPES)

Doutorando: Daniel Pens Gelain
Professores orientadores: Dr. José Cláudio Fonseca Moreira (Orientador no Brasil)
Dr. Peter Dunkley (Co-orientador no exterior)

INTRODUÇÃO

Este documento tem como objetivo apresentar um relatório referente ao período de estágio realizado na University of Newcastle, (Newcastle, Austrália), sob a supervisão do Prof. Peter Dunkley, financiado pela bolsa PDEE-CAPES. Este relatório irá conter uma descrição das condições de trabalho (a universidade e o laboratório, o grupo de pesquisa e adaptação ao ambiente de trabalho), dos experimentos realizados, com resultados, e das perspectivas abertas pelos resultados e pela experiência adquirida no exterior.

1. A Universidade de Newcastle e o Grupo de Neuroquímica

O Grupo de Neuroquímica da Universidade de Newcastle, coordenado pelo professor Peter Dunkley, há muitos anos vem recebendo alunos de doutorado e pós-doutorandos do Departamento de Bioquímica da UFRGS para a realização de estágios PDEE e de pós-doutorado. Além disso, o Prof. Peter Dunkley mantém colaborações ativas com m professores do Departamento de Bioquímica da UFRGS, realizando visitas constantes ao Brasil, tendo inclusive já residido em nosso país. Neste sentido, o processo de adaptação ao ambiente da Universidade foi absolutamente tranqüilo e não houve dificuldades, uma vez que o Prof. Peter Dunkley tem um bom conhecimento das demandas que um aluno vindo do exterior tem para adaptar-se inicialmente, tendo participado ativamente em todo o processo (busca de moradia, regularização de situação bancária, etc.). A cidade de Newcastle apresenta um ambiente favorável ao desenvolvimento deste tipo de atividade, por ser uma cidade universitária com um grande aporte de entrada de estudantes estrangeiros.

O Grupo de Neuroquímica é um grupo que tem um ótimo conceito na área, sendo um dos dois melhores grupos de estudo da regulação de TH no mundo, e também desenvolve trabalhos na área de sinalização celular em outros sistemas neuroquímicos. Todos os recursos utilizados pelo grupo foram disponibilizados em sua totalidade para a realização do trabalho previsto neste projeto, e houve um excelente entendimento com os outros membros do grupo. A participação em outros projetos de pesquisa do grupo foi estimulada, mas de forma que não atrapalhasse o andamento do projeto original, o que foi extremamente proveitoso. Não existe, de minha parte, nenhuma reclamação ou ressentimento em relação à recepção pelos professores, funcionários e outros estudantes da Universidade. Havia uma ampla infra-estrutura de apoio disponível para alunos de doutorado que ficou totalmente disponível para uso, compreendendo o uso da Biblioteca da Universidade, acesso livre à Internet pelos computadores da Universidade (a rede pode ser acessada em qualquer computador da Universidade através de uma senha individual), quotas livres de impressão a laser e de fotocópias, e um escritório com telefone e computador ligado à Internet a disposição. A secretaria também estava a disposição para solicitação de envio de fax para o exterior. Sendo assim, avalio que a Universidade de Newcastle apresenta condições extremamente favoráveis para a realização de projetos do PDEE-CAPES, sendo uma instituição totalmente adequada em todos os aspectos para o desenvolvimento deste tipo de atividade, tanto que os objetivos do projeto de pesquisa foram atingidos em sua totalidade, como veremos a seguir.

2. Resultados

No projeto original, havíamos proposto avaliar a modulação da fosforilação e atividade da TH em um modelo de cultura celular, as células PC-12, que é uma linhagem obtida de células cromafins da medula adrenal. Após o primeiro *set* de experimentos com estas células (incubando variadas concentrações de retinol entre 1 e 10 micromolar por variados tempos), nós observamos que a incubação com retinol interferia na regulação do ciclo de divisão destas células, e isto interferiu na fosforilação basal da TH. Sendo assim, nós decidimos mudar o modelo celular para culturas primárias de células cromafins bovinas (BACC), as quais são células diferenciadas e que não apresentam divisão celular.

2.1) Ativação da TH: Foi proposto avaliar o efeito da vitamina A (retinol) e de seu derivado metabólico mais relevante biologicamente, o ácido retinóico (RA), de induzir um aumento de fosforilação e conseqüente ativação da TH. Nós encontramos que o ácido retinóico não exerceu nenhum efeito na faixa de concentração testada (entre 0,1 e 10 micromolar), mas que o retinol exerceu um efeito de ativação da enzima, entre 5 minutos e 2 horas de incubação, de maneira dose-dependente. Este efeito foi demonstrado ser dependente da fosforilação dos resíduos ser40 e ser31 da extremidade amino-terminal da enzima, conforme avaliado por *western-blot*.

2.2) Rotas de sinalização envolvidas: Foi proposto originalmente que a rotas de sinalização envolvidas em um possível efeito da vitamina A sobre a ativação da TH seriam investigadas. Nós encontramos, através do uso de inibidores específicos e de anticorpos para as formas ativas de diversas kinases regulatórias, que a fosforilação da ser40 da TH causada pelo retinol é dependente da ativação da PKC por uma via dependente do influxo cálcio extracelular. Além disso, determinamos que PKA e PKG não estavam envolvidas neste efeito.

2.3) Envolvimento de Espécies Ativas de Oxigênio (ROS) na ação do retinol sobre as rotas de sinalização reguladoras da TH: Havíamos observado, durante o período prévio ao estágio, que o aumento na produção de ROS levava à ativação de kinases como Src e MEK1/2 em células de Sertoli. Assim, nós testamos o efeito do retinol e de ácido retinóico na produção de ROS em BACC, e observamos que retinol induziu um aumento constante na produção de ROS em BACC ao longo do tempo de incubação. Este aumento de ROS foi observado ser responsável pela ativação das ERK 1/2 e da fosforilação da ser31 da TH em BACC, como descrito a seguir.

2.4) Fosforilação da TH via MAPKs: Observamos que a fosforilação da ser31 e sustentação da ativação da TH por períodos maiores que 1 hora foi dependente da ativação das ERK1/2. Ativação de JNK e p38 não foi observada em nenhum período testado. Esta ativação foi revertida por quelantes de cálcio.

2.5) Envolvimento de radicais mediando as ações do retinol sobre MAPKs e fatores de transcrição: Nós propusemos, originalmente, relacionar a produção de ROS com a ativação de rotas de sinalização envolvidas na fosforilação e ativação da TH. Nós observamos que antioxidantes reverteram o efeito do retinol sobre a ativação das ERK 1/2 e da fosforilação da ser31 da TH. Além disso, a produção de ROS foi observada ser

dependente do influxo de cálcio extracelular. Não foi encontrado o envolvimento de fatores de transcrição neste efeito, sugerindo a presença de um efeito não-genômico.

Está sendo elaborado, neste momento, um artigo científico reportando os utilizados descritos acima, o qual está em fase final de elaboração; além disso, dois outros artigos[1,2] foram publicados contendo dados provenientes do trabalho realizado pelo bolsista.

[1]**PACAP stimulates the sustained phosphorylation of tyrosine hydroxylase at serine 40;** *Cellular Signalling*, **in press; doi:10.1016/j.cellsig.2006.12.006**

[2]**Cadmium stimulates MAPKs and Hsp27 phosphorylation in bovine adrenal chromaffin cells; Toxicology, in press; doi:10.1016/j.tox.2007.01.023**

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2 April, 2007

To Whom It May Concern:

Daniel Pens Gelain has recently finished working in my laboratory. He has been on a "Sandwich" year funded by CAPES. Daniel was an excellent scientist and representative of his country. He worked extremely hard, he was successful in all that he did and he had a very productive year. All of the staff here enjoyed interacting with him because of his professional approach to science, his cheerful disposition and his willingness to help. Daniel worked on four projects while he was here:

The first and most important was his study of the effects of retinoids on tyrosine hydroxylase. This was a major study and Daniel did all of the laboratory work. The study involved use of bovine adrenal chromaffin cells and an analysis of the signal transduction pathways activated by retinol and their effects on intracellular calcium and the activity and phosphorylation of tyrosine hydroxylase. Daniel completed this study within one year. This was a remarkable achievement considering that he had to learn the tyrosine hydroxylase activity and phosphorylation assays, establish the FURA 2 and the ROS assays and do it all by himself. Daniel has now completed a draft of the paper and has completed the figures. This paper will be submitted in the very near future to the top neurochemistry journal.

The second project involved helping us to complete a project that had been ongoing in the laboratory before his arrival. Daniel played a substantial role in this project completing work that was included in the final three figures of the paper. This involved establishing the PKA and PKC substrate antibody procedures and evaluating the PKA agonist effects in bovine adrenal chromaffin cells. This paper has now been published in a high impact journal.

Bobrovskaya, L., **Gelain, D. P.**, Gilligan, C., Dickson, P.W. and Dunkley, P. R. (2007) PACAP stimulates the sustained phosphorylation of tyrosine hydroxylase at serine 40. **Cellular Signalling.** In Press (CLS-D-06-00341) (SCI 4.4)

The third project involved undertaking some critical experiments for a paper that had been an ongoing collaboration between my laboratory and Rodrigo Leal's laboratory in Brazil. The referee of the paper had insisted on us undertaking further work before the paper would be accepted. Daniel completed these studies which became a figure in the paper. This work has now been published in a high quality journal.

Leal, R. B., Posser, T., Rigon, A. P., Oliveira, C. S., Goncalves, C. A., **Gelain, D. P.** and Dunkley, P. R. (2007) Cadmium stimulates MAPKs and HSP27 phosphorylation in bovine adrenal chromaffin cells. Toxicology, In Press. (SCI 2.6)

The fourth project involved evaluating a range of cell lines as potential models for studies that will be completed in Brazil. These included PC12 cells, SHSY5Y cells and other dopaminergic lines. Daniel learned a number of techniques and has a range of basic information that he can now use.

Peter Dunkley
Professor of Medical Biochemistry.

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