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O papel do retinol sobre a modulação do receptor para produtos finais avançados de glicação (RAGE) em linhagem de câncer de pulmão de não-pequenas células.

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PARTE I

RESUMO

A vitamina A e seus derivados, os retinóides, participam de processos celulares tais como desenvolvimento, proliferação, diferenciação e apoptose. Os efeitos da vitamina A, em sua maior parte, são atribuídos à ativação de receptores nucleares da família dos receptores esteróides, conhecidos como receptores de ácido retinóico (RAR) e receptores de retinóides (RXR). No entanto, o excesso de vitamina A, ou de retinóides, na dieta, ou devido a uso terapêutico, pode ser teratogênico. Diversos trabalhos tem demonstrado "*in vitro*" e "*in vivo*" que a vitamina A e seus metabólitos são moléculas redox ativas, isto é, podem agir como pró-oxidantes ou antioxidantes, dependendo da concentração e do ambiente em que se encontram. Nesse trabalho nós demonstramos que o tratamento com retinol, o qual é considerado o principal metabólito da vitamina A, em células da linhagem A549, a qual é derivada de um carcinoma de pulmão de não-pequenas células, levou a uma diminuição da expressão e do imunoconteúdo do receptor para produtos finais avançados de glicação (RAGE). O RAGE é constutivamente expresso nas células pulmonares, sendo que qualquer alteração na sua expressão ou imunoconteúdo induz alterações na homeostase do tecido pulmonar. Nós verificamos que a modulação do RAGE induzida pelo tratamento com retinol em nosso modelo foi redox dependente. O tratamento das células A549 com retinol induziu um aumento na produção de espécies reativas do oxigênio, o qual por consequência tornou o ambiente celular pró-oxidativo favorecendo a indução de danos a biomoléculas como lipídeos e proteínas. Além disso, o aumento na produção de espécies reativas do oxigênio levou a ativação da MAPK p38, por um mecanismo redox-dependente. A ativação da p38 subsequentemente levou à ativação do fator de transcrição NF-kB, o qual por consequência acabou por modular a expressão e imunoconteúdo do RAGE nessas células. Todos os efeitos ocasionados pelo tratamento com retinol foram bloqueados com o co-tratamento com Trolox® (análogo hidrofílico da vitamina E), o qual é uma molécula antioxidante. Portanto, nossos resultados demonstraram que o tratamento com retinol é capaz de induzir um estado pró-oxidativo nas células A549, o qual por consequência acaba por modular a expressão do RAGE de maneira redox-dependente e via a ativação da rota de sinalização da p38 - NF-kB. Então, a partir destes resultados e de outros dados já reportados na literatura, acreditamos que é necessário uma maior cautela no uso de vitamina A tanto a nível de suplementação quanto a nível terapêutico.

ABSTRACT

Vitamin A and its derivatives, the retinoids, participate in cellular processes such as growth, cell division and apoptosis. The effects of vitamin A, in part, are ascribed to gene transcription mediated by nuclear receptors from the family of the steroid receptors, known as retinoic acid receptors (RARs) and retinoid receptors (RXRs). However, excessive vitamin A, or retinoids, in the diet, or by therapeutic use, may be theratogenic. Several studies have shown "in vitro" and "in vivo" that vitamin A and its metabolites are redox active molecules, that is, may act as pro-oxidants and antioxidants, depending on the concentration and the environment in which they are. In this study we demonstrated that treatment with retinol, which is considered to be the principal metabolite of vitamin A, in cell lines of non-small cells lung carcinoma A549 led to a decrease in the expression and immunocontent of receptor for advanced glycation endproducts (RAGE). The RAGE is constitutively expressed in lung cells; a modulation in expression or immunocontent of RAGE is associated with alterations on homeostasis of lung tissue. Here, we found that the modulation of RAGE induced by retinol treatment in our model was redox dependent. Retinol treatment of A549 cells induced an increase in the production of reactive oxygen species, which consequently become the pro-cellular environment favoring the induction of oxidative damage to biomolecules such as lipids and proteins. Furthermore, the increase in production of reactive oxygen species led to activation of p38 MAPK by a redox-dependent mechanism. Activation of p38 subsequently led to activation of the transcription factor NF-kB, which consequently modulates the expression of RAGE in these cells. All effects caused by retinol treatment were blocked with Trolox ® (hydrophilic analogue of Vitamin E) co-treatment, which is an antioxidant molecule. Therefore, our results demonstrated that retinol treatment is able to induce a pro-oxidative status in A549 cells, and that modulation of RAGE expression by retinol is mediated by the redox-dependent activation of p38/NF-kB signaling pathway. Then, based on these results and other data already reported in the literature, we believe that greater care is needed in the use of vitamin A supplementation as therapeutic level.

LISTA ABREVIATURAS

AGE - Produtos finais avançados de glicação

ARAT- Acil-CoA:retinol aciltransferase

CAT- Catalase

CRALBP – Proteína celular ligadora de retinaldeído

CRABP – Proteína celular ligadora de ácido retinóico

CRBP – Proteína celular ligadora de retinol

CREB – Proteína ligadora de elemento responsivo a nucleotídeo cíclico

CYP26- Citocromos P450 da família 26

Cu^+/Zn^+-SOD – Superóxido dismutase cobre/zinco

DNA – Ácido desoxirribonucléico

ERK ½- Cinases reguladas por sinal extracelular 1 e 2

ERO- Espécies reativas do oxigênio

GSH- Glutationa redutase

GPx- Glutationa peroxidase

H_2O_2 – Peróxido de hidrogênio

JNK – Jun cinase

LIC - Células intersticiais lipídicas

LPS - Lipopolissacarídeo

LRAT – Lecitina:retinol aciltransferase

MAPK - Proteína cinase ativadora mitogênica

NF-kB - Fator nuclear kB

$NO^{\bullet}$ - Óxido nítrico

$O_2^{\bullet -}$ - Ânion superóxido

$OH^{\bullet}$ - Radical hidroxila

$ONOO^-$ Peroxinitrito

ONOOH - Nitrosila

p38 MAPK - Proteína cinase ativadora mitogênica p38

RAGE - Receptor para produto final avançado de glicação

RAR – Receptor de ácido retinóico

RALDH – Retinal desidrogenase

RBP – Proteína ligadora de retinol

REH- Retinol éster desidrogenase

RXR – Receptor retinóide X

siRNA - Pequeno ácido ribonucléico de interferência

1. INTRODUÇÃO

1.1 VITAMINA A E OS RETINÓIDES

A vitamina A (retinol) e seus metabólitos como: retinal, ésteres de retinil, e ácido retinóico, são conhecidos coletivamente como retinóides (Bollag 1983). Nos organismos eles exercem um importante papel em processos como o crescimento e diferenciação celular (Gudas 1994; Rogers 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 visão e funções motoras (Wang, Cui et al. 2012; Zhong, Kawaguchi et al. 2012).

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: álcool, aldeído ou ácido (Figura 1) (Bollag 1983). O termo geral de retinóides compreende então o retinol, o qual é considerado a "molécula-raiz" 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 os ésteres de retinil e retinal. Além disso, vários análogos sintéticos com similaridade estrutural ou funcional ao retinol também são reconhecidos como pertencentes à grande família dos retinóides (Zhong, Kawaguchi et al. 2012).

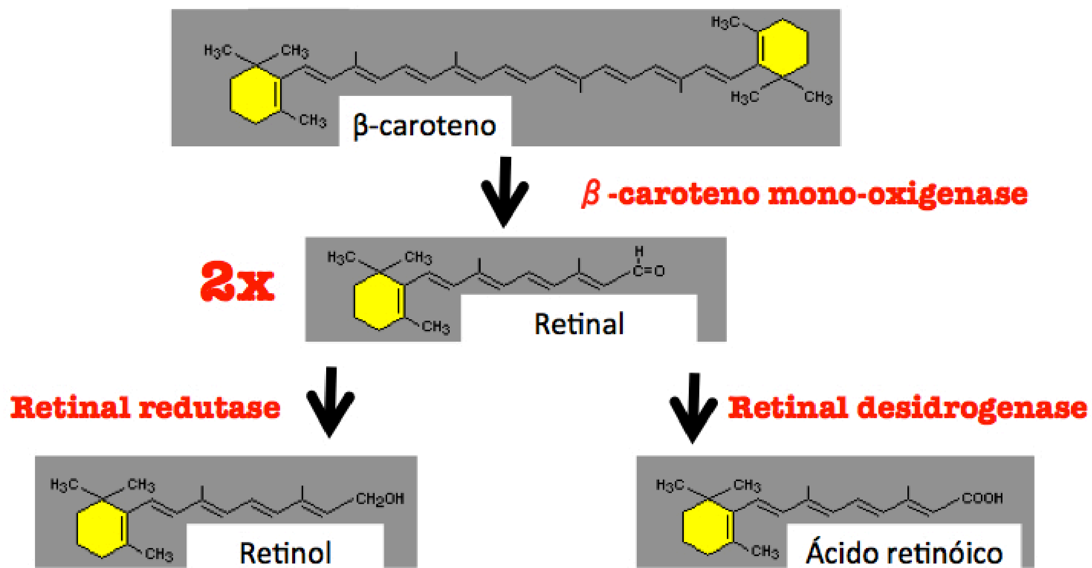


Figura 1 - Estrutura do β-caroteno o qual é a molécula precursora dos retinóides, juntamente com as três principais configurações isoméricas da vitamina A

A síntese *de novo* de retinol é restrita às plantas e a alguns microorganismo (Mercer, Davies et al. 1963). Animais obtém 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 (von Lintig 2012). O beta-caroteno, por exemplo, é convertido em retinol através de dois passos enzimáticos, na mucosa intestinal. Por outro lado, a principal forma de vitamina A pré-formada encontrada na dieta é o retinol esterificado a ácido graxos de cadeia longa (chamados genericamente de ésteres de retinol), obtido através de alimentos de origem animal (principalmente fígado, leite e derivados) (Olson 1996), além de alimentos processados industrialmente e em suplementos vitamínicos.

Após a ingestão, tanto o beta-caroteno como os ésteres 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 da mucosa 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 e 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 quilomícra, e transportados através do sistema linfático (Harrison e Hussain 2001; Schweigert e Raila 2002).

Durante o processamento dos quilomícra pelos tecidos extra-hepáticos, algumas células obtém ésteres de retinol liberados pela ação da lipase lipoprotéica (Miano e Berk 2000). No tecido pulmonar os ésteres de retinol são obtidos por células denominadas de células intersticiais lipídicas (Células intersticiais lipídicas, LIC) . As LICs estão localizadas entre a parede das células dos capilares alveolares e as células endoteliais dos capilares, sendo elas responsáveis por armazenar o retinol no tecido pulmonar (Kaplan, Grant et al. 1985). Os quilomícra remanescentes são, em seguida, captados pelo fígado, que é o principal órgão armazenador de vitamina A sob condições normais (Yost, Harrison et al. 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 (Proteína celular ligadora de retinol ou CRBPs) (Thompson e 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 (Proteínas ligadoras de retinol, RBPs), nos hepatócitos, para secreção na circulação (Soprano, Gyda et al. 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 (Harrison e Hussain 2001; Schweigert e Raila 2002) 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 proteínas celulares ligadoras de retinaldeído , CRALBPs) ou ser oxidado a ácido retinóico pela retinal desidrogenase (RALDH). O ácido retinóico citoplasmático também se encontra associado a proteínas ligadoras específicas (Proteínas ligadoras de ácido retinóico, 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 devido ao fato que quando na sua forma de ácido retinóico 9-*cis* ele interaja com os receptores nucleares de retinóides ao invés de interagir com os receptores nucleares de ácido retinóico (Elliott 2005).

1.2 MECANISMOS DE AÇÃO

Em 1987, descobriu-se que os retinóides exercem suas funções através da sua ligação a receptores nucleares específicos (receptores de retinoides, RXR; e receptores de ácido retinóico, RAR) que, por sua vez, agem como fatores de transcrição, regulando, portanto, a expressão gênica de sequências de DNA alvo àquelas moléculas sinalizadoras. Por esta razão, estes compostos têm sido definidos, por muitos autores, como hormônios (Petkovich, Brand et al. 1987).

Além da forma tradicionalmente aceita de sinalização mediada por vitamina A, têm se demonstrado que a vitamina A pode atuar, em nível celular, de maneira independente do núcleo, por meio de uma 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) (Elliott 2005). No entanto, isso não significa que esses receptores não estão envolvidos nesse fenômeno, como sugerem alguns autores (Ghyselinck, Dupe et al. 1997; Piskunov e Rochette-Egly 2012). 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 regulação 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 p38 MAPK, cdk

e JNK (Bastien e Rochette-Egly 2004). Nosso grupo também demonstrou que a via de sinalização celular ERK ½ -CREB pode ser ativada por retinol em concentrações pouco acima das fisiológicas no modelo experimental utilizado (Gelain, Cammarota et al. 2006). Portanto, essas ações não-genômicas da vitamina A parecem independer de sua ligação a receptores nucleares, mas o exato mecanismo de ação ainda merece ser investigado.

1.3 RADICAIS LIVRES E ESTRESSE OXIDATIVO

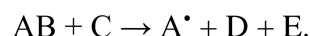
Um radical livre é uma espécie química com um ou mais elétrons desemparelhados no seu último orbital. As espécies químicas podem ser átomos, como hidrogênio ou cloreto; metais de transição; ou uma molécula onde o elétron desemparelhado esteja localizado no orbital externo. Este elétron desemparelhado confere uma reatividade relativamente alta a esta molécula, devido a uma grande tendência de esta adquirir um segundo elétron para este orbital (Gutteridge e Halliwell 2000).

Radicais livres são escritos quimicamente com uma notação para a espécie química seguida de um ponto, o qual indica o elétron desemparelhado, por exemplo, o radical livre ânion superóxido: $O_2^{-\bullet}$.

Quimicamente, radicais livres são caracterizados por sustentarem reações em cadeia, que se autopropaga, onde uma molécula reduzida perde seu elétron para o radical livre, e aquela reduzida se torna, agora, um radical livre, reagindo com outro composto químico, e assim por diante.

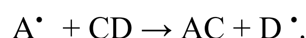
Classicamente, as reações de radicais livres são divididas em: a) reações de iniciação; b) reações de propagação; e c) reações de terminação. Nas reações de

iniciação, um radical livre é formado a partir de espécies químicas não-radicais (e, portanto estáveis):



$$AB + C \rightarrow A^{\bullet} + D + E.$$

Nas reações de propagação, um radical livre, também chamado centro de reação, reage com uma molécula estável, resultando em outro radical livre, ou centro de reação:



$$A^{\bullet} + CD \rightarrow AC + D^{\bullet}.$$

Nas reações de terminação, dois radicais livres cancelam seus elétrons desemparelhados formando um produto estável:

$$A^{\bullet} + B^{\bullet} \rightarrow AB.$$

A reatividade química dos radicais livres é determinada pela molécula que carrega este elétron desemparelhado; consequentemente, a reatividade varia muito entre um radical e outro. Um modo de expressar e comparar a reatividade química destas moléculas é especificar a meia-vida (t ½) das mesmas. Uma meia-vida curta indica alta reatividade, e o radical hidroxila ($OH^{\bullet}$) é o mais reativo dos radicais livres (sendo, então, o mais instável, ou seja, é aquele que reage mais rapidamente assim que formado).

O radical livre de ocorrência mais comum é o ânion superóxido ($O_2^{-\bullet}$), que é produzido quando uma molécula de oxigênio é reduzida parcialmente, ou seja, quando recebe apenas um elétron, ao invés de receber dois elétrons. Quantidades excessivas de $O_2^{-\bullet}$ levam a dano tecidual por induzir a produção de hidroxila ($OH^{\bullet}$) derivado da reação de peróxido de hidrogênio (H_2O_2) presente no ambiente celular com metais de transição Fe^{2+} ou Cu^{2+} através da reação de Fenton. Além disso, ao

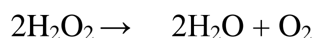
reagir com óxido nítrico ($NO^{\bullet}$), o $O_2^{-\bullet}$ forma peroxinitrito ($ONOO^-$) que, por sua vez, pode gerar o radical nitrosila (ONOOH) que, ao se decompor, também forma o radical hidroxila ($OH^{\bullet}$).

Os radicais livres podem causar dano oxidativo aos componentes celulares como lipídios, carboidratos, proteínas e DNA devido à sua alta reatividade e natureza oxidante. No entanto, as células contam com defesas contra tais efeitos danosos gerados pelos radicais livres. São as defesas antioxidantes, que podem ser tanto enzimáticas quanto não-enzimáticas (Gutteridge e Halliwell 2000). Entre as defesas enzimáticas estão as enzimas superóxido dismutase, catalase e glutationa peroxidase. O tripeptídio glutationa (na forma reduzida – GSH) e as vitaminas (originadas da dieta, como o ácido ascórbico, e a vitamina E, por exemplo) representam defesas antioxidantes não-enzimáticas.

As enzimas anteriormente citadas são tidas como defesas antioxidantes primárias, ou seja, agem diretamente sobre a molécula do radical livre, antes que este possa vir a oxidar uma biomolécula. A enzima superóxido dismutase (SOD) apresenta quatro classes: Mn-SOD (localizada na matriz mitocondrial), Cu, Zn-SOD (citosólica), Ni-SOD e SOD extracelular. Todas estas formas de SOD agem sobre o radical $O_2^{-\bullet}$, transformando-o em peróxido de hidrogênio (H_2O_2) e oxigênio através da seguinte reação:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

Já a enzima catalase (CAT), age sobre o H_2O_2 gerado na reação acima, transformando-o em água por meio da reação:



$$2H_2O_2 \rightarrow 2H_2O + O_2$$

A enzima glutationa peroxidase (GPx) também atua sobre o H_2O_2, no entanto, por meio de um mecanismo diferente. A GPx participa de um ciclo redox junto da enzima glutationa redutase, onde GSH é usada pela GPx para transformar H_2O_2 em água; e NADPH é utilizado pela glutiona redutase para reduzir a glutationa oxidada, produto da primeira reação, em GSH novamente (Boveris 1998).

Estresse oxidativo é o termo utilizado em uma situação onde a formação de radicais livres excede a capacidade de transformação destas moléculas em outras não oxidantes por meio das defesas antioxidantes. Neste caso, podemos dizer que ocorreu um desequilíbrio entre a formação de radicais livres e a atuação da defesa antioxidante. E isto pode ocorrer por diversos motivos, inclusive, inativação de enzimas como SOD e CAT por meio de reações destas com as próprias moléculas oxidantes, onde as enzimas perdem sua característica nativa e, consequentemente, sua função. Um exemplo é a inativação de enzima CAT por $O_2^{-\bullet}$ (Shimizu, Kobayashi et al. 1984) .

Dentro da célula, podem-se encontrar fontes de formação de radicais livres, tais como a cadeia transportadora de elétrons mitocondrial, onde a redução parcial do oxigênio dará origem ao $O_2^{-\bullet}$. Existem dois locais da cadeia transportadora de elétrons de onde os elétrons podem vazar, formando o $O_2^{-\bullet}$. O primeiro é a partir da NADH desidrogenase (Complexo I) e o outro é a partir do Complexo III.

Embora a mitocôndria seja o local mais importante de produção de radicais livres endógenos, existem outros locais onde estas moléculas oxidantes podem ser formadas. No citosol, por exemplo, a cascata do ácido araquidônico, que produz prostaglandinas e leucotrienos, pode formar espécies reativas do oxigênio (ERO)

quando o lipídio metabolizado é liberado. Ainda, algumas isoenzimas citocromo P-450 também estão descritas como produtoras de ERO (Gutteridge e Halliwell 2000).

1.4 VITAMINA A E O TECIDO PULMONAR

A vitamina A influencia diretamente a maturação e diferenciação das células pulmonares. As células alveolares do tipo II são as células que secretam surfactante. Surfactante é uma mistura de fosfolipídios com proteínas, o qual tem como função diminuir a tensão superficial das células pulmonares. A expressão de proteínas surfactantes pelas células alveolares do tipo II é regulada de maneira dose-dependente por ácido retinóico. Além disso, a vitamina A é conhecida por preservar e manter a integridade do epitélio pulmonar. Durante episódios de injúria, a vitamina A é responsável pela proliferação das células alveolares do tipo II e de sua diferenciação em células alveolares do tipo I (Takahashi, Miura et al. 1993).

Já foi relatado que a deficiência em vitamina A leva a uma diminuição no conteúdo de elastina nos pulmões, diminui a síntese de surfactante pelas células alveolares do tipo II, formação de áreas com líquido intersticial, efeitos esses que estão ligados diretamente a processos patológicos de doenças pulmonares (McGowan, Takle et al. 2005). Entretanto, pouco se sabe sobre os possíveis efeitos da suplementação com vitamina A (palmitato de retinol) em relação ao tecido pulmonar no que diz respeito ao ambiente redox deste. Alguns estudos observaram que a suplementação com vitamina A em doses consideradas terapêuticas pode aumentar o risco de morte entre pacientes fumantes que possuam câncer de pulmão (Omenn, Goodman et al. 1996). Recentemente, nosso grupo de pesquisa demonstrou que a suplementação de vitamina A (palmitato de retinol) por diferentes períodos (3, 7 e 28 dias) em doses consideradas terapêuticas (1.000, 2.500, 4.500, e 9.000 U.I./kg.dia^{-1})

causa alteração no perfil redox do tecido pulmonar de ratos Wistar (Pasquali, Gelain et al. 2009a; Pasquali, Gelain et al. 2009b). Mais especificamente, observamos que a suplementação induz um aumento de danos oxidativos a biomoléculas como lipídeos e proteínas, alteração da atividade das enzimas antioxidantes e diminuição do conteúdo do receptor para produtos finais de glicação avançada (RAGE) deste tecido. No entanto, ainda não está claro quais são as reais consequências para o tecido pulmonar destes efeitos observados com a suplementação com vitamina A.

1.5 RECEPTOR PARA PRODUTOS FINAIS DE GLICAÇÃO AVANÇADA *(RAGE)*

O receptor para produtos finais de glicação avançada (RAGE) é uma proteína transmembrana que pertence a família das imunoglobulinas, o qual serve com padrão de reconhecimento do sistema imune inato. O RAGE foi caracterizado a cerca de 20 anos atrás, sendo que primeiramente ele foi extraído do pulmão de bovinos (Schmidt, Hofmann et al. 2000). Inicialmente ele foi identificado como um receptor para produtos finais avançados de glicação (AGEs), os quais são um grupo heterogêneo de compostos formados no corpo espontaneamente por meio de reações não-enzimáticas entre açucares não-redutores, proteínas e lipídeos; ou obtidos através da dieta pela ingestão de alimentos enriquecidos em AGEs. Porém, estudos recentes já descreveram que outros peptídeos endógenos possuem capacidade de se ligar ao RAGE , como a proteína anfoterina HMGB-1, proteínas da família S100/calgranulina, fibrilas amilóides, lipopolisacarídeo (LPS) e fosfatidilserina (Schmidt e Stern 2000).

Com exceção dos pulmões, onde sua expressão é alta, o RAGE é muito pouco expresso e detectado em outros tecidos (Hsieh, Schafer et al. 2003). Na maioria dos outros tecidos, quando detectado, o RAGE parece estar envolvido na resposta destes tecidos a processos como de injúria, infecção ou inflamação (Hsieh, Schafer et al.

2003). Nestes processos, o RAGE através da interação com seus ligantes, induz repostas intracelulares mediante a ativação de vias de sinalização como NF-kB, CREB e STAT3. A ativação destas vias de sinalização acabam por regular a transcrição de genes envolvidos na sobrevivência, proliferação, diferenciação, migração, fagocitose e autofagia celular no tecido onde o RAGE está sendo ativado.

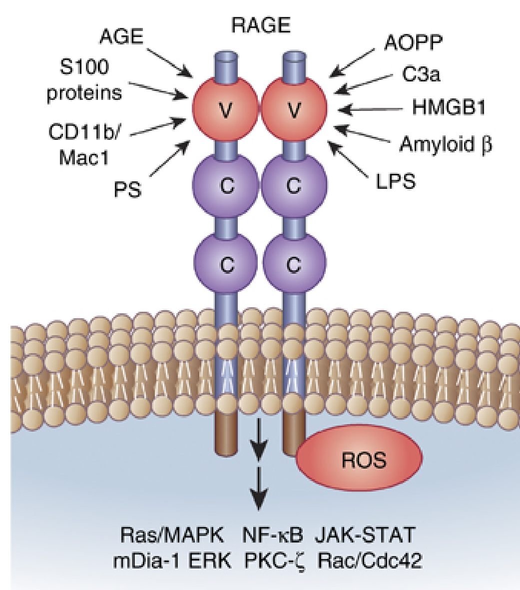


Figura 2 - Representação do receptor para produtos finais de glicação avançada e dos seus possíveis ligantes e vias de sinalização que o mesmo pode levar a ativação.

No tecido pulmonar, especula-se que alta expressão do RAGE seja para manter a homeostasia do tecido pulmonar (Queisser, Kouri et al. 2008). Recentemente vêm sendo sugerido a utilização da expressão de RAGE para diagnóstico de doenças respiratórias assim como o seu uso no tratamento das mesmas e isso se deve ao fato de ele ter sua expressão diminuída nas doenças respiratórias (Arancio, Zhang et al. 2004; Hudson e Schmidt 2004). Sabe-se também que a diminuição da expressão de

RAGE assim como o seu polimorfismo está associada ao aumento de proliferação das células pulmonares e incidência de câncer respectivamente (Wang, Cui et al. 2012).

A regulação de RAGE nos pulmões ainda é muito pouco conhecida, principalmente devido ao fato de sua regulação ser tecido específica. Em nossos trabalhos de suplementação com vitamina A em ratos Wistar, nós observamos que o RAGE é modulado de forma tecido específica. No tecido pulmonar esse receptor tem seu conteúdo diminuído com a suplementação com vitamina A, enquanto que em tecidos cerebrais ocorre um aumento no seu conteúdo (de Oliveira, Oliveira et al. 2009).

2. JUSTIFICATIVA

Alguns dados da literatura, incluindo trabalhos do nosso grupo, mostram que a vitamina A, e alguns de seus derivados, são moléculas redox ativas, ou seja, dependendo de algumas condições, podem reduzir ou oxidar outras biomoléculas. Dentre os efeitos observados com a suplementação com vitamina A, temos aumento na produção mitocondrial de radical superóxido, aumento nos níveis de marcadores de peroxidação lipídica e de carbonilação de proteínas, e modulação na atividade de enzimas antioxidantes, tais como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Além desses dados envolvendo o balanço redox, também foi observado que a suplementação com vitamina A pode levar alterações nos níveis de RAGE. Estes dados mostram que, em diferentes modelos experimentais, o tratamento com retinol/palmitato de retinol pode ocasionar alterações redox as quais por consequência induzem estresse oxidativo (Gelain, Cammarota et al. 2006; de Oliveira, Oliveira et al. 2009; Pasquali, Gelain et al. 2009a; Pasquali, Gelain et al. 2009b; Pasquali, Schnorr et al. 2010).

3. OBJETIVOS

A partir dos resultados previamente publicados, decidimos elucidar o mecanismo pelo qual o retinol modula o receptor de produtos finais avançados de glicação (RAGE) em células da linhagem tumoral A549, a qual é uma linhagem de carcinoma de pulmão de não-pequenas células.

Como objetivos específicos para elucidarmos o mecanismo pelo qual a retinol modula o RAGE nós investigamos o tratamento das células A549 com retinol em concentrações de 2, 5, 10 e 20 μM, as quais são concentrações fisiológicas e supra-fisiológicas, e avaliar:

- Perfil redox das células após o tratamento, avaliando parâmetros como: peroxidação lipídica, carbonilação de proteínas, estado redox de grupamentos sulfridril protéicos e não-protéicos, atividade das enzimas antioxidantes: superóxido dismutase (SOD) e catalase (CAT);
- Produção de espécies reativas, utilizando como parâmetros: produção mitocondrial de radical superóxido, produção de radical superóxido pela enzima NADPH-oxidase, produção de espécies reativas e viabilidade celular; e sua participação na modulação do RAGE;
- Modulação do receptor para produtos finais avançados de glicação (RAGE);
- Ativação de vias de sinalização das proteínas cinases ativadoras mitogênicas (MAPK) - p38, ERK 1/2, e JNK e sua participação na modulação do RAGE;
- Ativação do fator de transcrição NF-kB e sua participação na modulação do RAGE.

PARTE II

CAPÍTULO I

"Vitamin A (retinol) downregulates the receptor for advanced glycation endproducts (RAGE) by oxidant-dependent activation of p38 MAPK and NF-kB in human lung cancer A549 cells"

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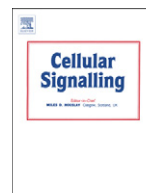

Vitamin A (retinol) downregulates the receptor for advanced glycation endproducts (RAGE) by oxidant-dependent activation of p38 MAPK and NF-kB in human lung cancer A549 cells

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ABSTRACT

As an essential component of the diet, retinol supplementation is often considered harmless and its application is poorly controlled. However, recent works demonstrated that retinol may induce a wide array of deleterious effects, especially when doses used are elevated. Controlled clinical trials have demonstrated that retinol supplementation increased the incidence of lung cancer and mortality in smokers. Experimental works in cell cultures and animal models showed that retinol may induce free radical production, oxidative stress and extensive biomolecular damage. Here, we evaluated the effect of retinol on the regulation of the receptor for advanced glycation end-products (RAGE) in the human lung cancer cell line A549. RAGE is constitutively expressed in lungs and was observed to be down-regulated in lung cancer patients. A549 cells were treated with retinol doses reported as physiologic (2 μM) or therapeutic (5, 10 or 20 μM). Retinol at 10 and 20 μM increased free radical production, oxidative damage and antioxidant enzyme activity in A549 cells. These doses also downregulated RAGE expression. Antioxidant co-treatment with Trolox®, a hydrophilic analog of α-tocopherol, reversed the effects of retinol on oxidative parameters and RAGE downregulation. The effect of retinol on RAGE was mediated by p38 MAPK activation, as blockade of p38 with PD169316 (10 μM), SB203580 (10 μM) or siRNA to either p38α (MAPK14) or p38β (MAPK11) reversed the effect of retinol on RAGE. Trolox also inhibited p38 phosphorylation, indicating that retinol induced a redox-dependent activation of this MAPK. Besides, we observed that NF-kB acted as a downstream effector of p38 in RAGE downregulation by retinol, as NF-kB inhibition by SN50 (100 μg/mL) and siRNA to p65 blocked the effect of retinol on RAGE, and p38 inhibitors reversed NF-kB activation. Taken together, our results indicate a pro-oxidant effect of retinol on A549 cells, and suggest that modulation of RAGE expression by retinol is mediated by the redox-dependent activation of p38/NF-kB signaling pathway.

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1. Introduction

Retinol (vitamin A) and its metabolites (retinoids) are important micronutrients that regulate many biological processes such as cellular growth and differentiation [1,2]. The classical mechanism of action by retinoids is through activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR), which act as transcription factors. In the lungs, vitamin A is required for fetal development and alveolar septation [2]. During the postnatal period, it participates in the process of tracheal and bronchopulmonary tree formation. The lungs are formed by two types of cells: the type I cell and the type II cell. The type I cells are responsible for forming the barrier between the air and the capillary. The type II cells are especially prepared to synthesize and secrete lung surfactant protein. The expression of surfactant protein is regulated by vitamin A [3].

In the latest years many studies have described a protective role of vitamin A in several diseases related to lung development (e.g., asthma, chronic obstructive pulmonary disease, and parenchymal lung diseases). These studies exploit the ability of vitamin A as a scavenger of toxic metabolites widely known as free radicals [4–6]. Free radicals may exist as reactive oxygen species (ROS), reactive nitrogen species (RNS), and xenobiotic species. In many developing countries where infant malnutrition is a common problem, children are provided with a single high-dose of vitamin A supplement (300 to 10,000 IU/kg) at regular intervals, with various coverage rates. The World Health Organization (WHO) recommends that adults ingest an average of 5000 IU/day of vitamin A on their diet. The physiological range of retinol in cells varies between 0.2 and 5 μM [7,8]. Additionally, vitamin A has been

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recommended in a wide range of doses for treatment of some conditions, mainly in the field of oncology [9]. However, some authors observed that vitamin A may induce toxic effects to different cell types [10,11]. Retinol and its derivatives may exert pro-oxidant effects which may cause oxidative damage, cell cycle disruption, transformation and/or cell death [11–16]. Previously, we observed that daily vitamin A oral supplementation increases several parameters of oxidative stress in rat lungs [17–20]. Besides, clinical trials observed an increase of the incidence of lung cancer and colorectal cancer in smokers and asbestos-exposed men that received oral supplementation with vitamin A and/or beta-carotene [21–26]. Therefore, there is increasing evidence that retinol and/or its metabolites may alter the physiological and functional homeostasis of the lungs. Lung cells express the receptor for advanced glycation end-products (RAGE), which was initially isolated, identified and localized on basolateral membranes [27,28]. RAGE is a multi-ligand, immunoglobulin-type transmembrane protein, which serves as one of the pattern-recognition receptors (PRRs) of the innate immune system. It was initially identified as a receptor for predominantly advanced glycation end-products (AGEs), a heterogeneous group of compounds either formed spontaneously in the body via non-enzymatic glycol-oxidative reactions between reducing sugars, proteins, and lipids, or obtained directly from the intake of AGE-rich food [29]. Later, it was noticed that RAGE also interacts with diverse non-glycated endogenous peptide ligands such as high mobility group box 1 protein (HMGB-1), S100/calgranulin family proteins, amyloid fibrils, LPS and phosphatidylserine [30].

RAGE is expressed in a variety of cell types during prenatal and postnatal development and repressed at completion of development, excepting in the lung cells. RAGE expression is constitutive in the lungs and high levels of RAGE expression during lung development and in adult pulmonary tissue suggest a likely beneficial function in lung morphogenesis and homeostasis [31]. Low levels of RAGE are associated with pathogenesis of lung cancer and are observed also in lung diseases, such as asthma, chronic obstructive pulmonary disease and parenchymal lung diseases [32–34]. Interestingly, in other tissues, the increase of RAGE levels and activation influences a wide range of pathological conditions such as diabetes, pro-inflammatory states and neurodegenerative processes. Thus, the outcome of RAGE signaling is strongly cell type and context dependent. When RAGE binds its ligands, it triggers the activation of a number of key signaling pathways, such as NF-kB and mitogen-activated protein kinase (MAPK). NF-kB is formed by homo or heterodimers comprising members of the Rel family of proteins (p50/p105, p52/p100, p65, c-Rel and RelB) which form, upon non-stimulated conditions, a ternary and inactive cytoplasmic complex by interacting with inhibitory proteins of the IkB family. The MAPK pathway comprises three subgroups: the extra-cellular signal-regulated kinases 1 and 2 (ERK 1/2), the p38 MAPK, and the stress-activated protein kinase or c-Jun N-terminal kinase (SAPK/JNK). The activation of these intracellular pathways by RAGE triggering can lead to production of inflammatory cytokines, activation of proteases and oxidative stress [28]. However, the molecular basis leading to the activation of these signaling pathways after RAGE/ligands interaction remains to be elucidated.

Due to the high exposure to oxygen, the lungs are susceptible to the attack of ROS and RNS. The imbalance between antioxidants defenses and ROS/RNS can induce oxidative stress in the lungs. Oxidative stress and disruption of RAGE levels have been implicated in the pathogenesis of a variety of pulmonary disorders including lung cancer. In the current study, we investigated the effect of retinol treatment on the RAGE levels and oxidative stress parameters in A549 cell line, a human lung adenocarcinoma cell line with alveolar type II-like properties. We report here, for the first time, that retinol decreases RAGE levels by a mechanism dependent on the generation of intracellular reactive species and the activation of p38 MAPK and NF-kB. These data suggest that RAGE downregulation may exert a role in the deleterious effects observed in some retinol supplementation therapies, especially in the lungs.

2. Materials and methods

2.1. Chemicals

All-trans retinol alcohol, 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT), sulphorhodamine B (SRB), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®), diphenyliodonium chloride (DPI), 4′-hydroxy-3′-methoxyacetophenone (apocynin), dithiothreitol, 2-thiobarbituric acid (TBA), 4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate (UO126) and culture analytical grade reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Protease inhibitor cocktail was purchased from Roche Products Limited (Welwyn Garden City, UK). SN50 was purchased from Enzo Life Science (Farmingdale, NY, USA). Electrophoresis/immunoblotting reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Antibodies against total and phospho-JNK (Thr183/Tyr185), total and phospho-p38 MAPK (Thr180/Tyr182), total and phospho-ERK (Thr202/Tyr204), p38β MAPK, p38α MAPK, p65, Lamin B, β-actin, were all purchased from Cell Signaling (Beverly, MA, USA) (Product numbers: 2339, 9211, 9218, 4764, 9087, 4970, 4967). Anti-RAGE antibody was purchased from AbCam (Cambridge, UK).

2.2. Cell culture

The human non-small cell lung cancer (NSCLC) cell line A549 was grown in RPMI-1640, 10% FBS and maintained at 37 °C in an atmosphere containing 5% CO_2. The media were supplemented with 1% penicillin/streptomycin.

2.3. Treatments

Retinol was dissolved in ethanol. Concentrated stocks were prepared immediately before experiments by diluting retinol into ethanol and determining final stock concentration by UV absorption; solution was kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition. All treatments were initiated by adding concentrated solutions to reach final concentrations in the well. The final ethanol concentration did not exceed 0.1% in any experiment.

2.4. Thiobarbituric acid-reactive species (TBARS)

The cells were plated onto 6-well plates. When the culture reached 80% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of treatment, the cells were collected and homogenized. As an index of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described [35]. In brief, the samples from 6 well plates were mixed with 0.6 mL of 10% trichloroacetic acid and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. Results are expressed as nmol of TBARS/mg of protein [36].

2.5. Measurement of protein carbonyls

The cells were plated onto 6-well plates. When the culture reached 80% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of treatment, the cells were collected and homogenized. The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described [37]. In

brief, proteins were precipitated by addition of 20% trichloroacetic acid and redissolved in dinitrophenylhydrazine, and the absorbance was read in a spectrophotometer at 370 nm. Results are expressed as nmol of carbonyl/mg of protein.

2.6. Measurement of protein thiol content

The cells were plated onto 6-well plates. When the culture reached 80% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of treatment, the cells were collected and homogenized. Protein thiol content in samples was analyzed to estimate oxidative alterations in proteins [38]. In brief, an aliquot was diluted in 0.1% sodium dodecyl sulfate, 0.01 M 5,50-dithionitrobis(2-nitrobenzoic acid) in ethanol was added, and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min. Results are expressed as mmol of SH/mg of protein.

2.7. Estimation of antioxidant enzyme activities

The cells were plated onto 6-well plates. When the culture reached 80% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of treatment, the cells were collected and homogenized. Catalase (EC 1.11.1.6) (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm, and the results are expressed as units of CAT/mg of protein [39]. Bubble formation in oxygen generation by CAT activity was monitored and did not interfere with measurement of CAT activities in the linear range used to measure CAT activity. Superoxide dismutase (EC 1.15.1.1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described, and the results are expressed as units of SOD/mg of protein [40]. A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of retinol treatment on these two oxidant detoxifying enzymes that work in sequence converting superoxide anion to water [20]. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

2.8. Determination of intracellular RS production (real-time dichlorofluorescein oxidation assay)

Intracellular reactive species production was determined by the DCFH-DA-based real-time assay using intact living cells. Briefly, A549 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 [41]. 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 intracellular reactive species 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 Graphpad 5.0 software.

2.9. MTT assay

The cells were plated onto 96-well plates. When the culture reached 60% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of retinol treatment, A549 cell 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 0.5 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_2 300 μM was used as positive control for cell death.

2.10. Sulphorhodamine B (SRB) assay

This colorimetric assay was performed to assess growth. It estimates cell numbers indirectly by staining total cellular protein with SRB [42]. The cells were plated onto 96-well plates. When the culture reached 60% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of retinol treatment, cells were fixed by layering 100 μL of ice-cold 40% trichloroacetic acid (TCA) on top of the growth medium and incubated at 4 °C for 1 h. Plates were then washed five times with cold water. The excess water was then decanted and the plates left to dry in air. SRB stain (50 μL; 0.4% in 1% acetic acid) was added to each well and left in contact with the cells for 30 min. The cells were then washed with 1% acetic acid, rinsed 4 times until only the dye adhering to the cells was left. The plates were then air-dried and 100 μL of 10 mM Tris base pH 10.5 was added to each well to solubilize the dye. The plates were gently shaken for 20 min on a gyratory shaker and the absorbance (OD) of each well was read at 492 nm. Cell survival was measured as the percentage absorbance compared to the absorbance of a control (non-treated cells).

2.11. Superoxide radical anion production

To quantify the superoxide radical anion ($O_2^{-\bullet}$) production, we isolated submitochondrial particles (SMPs) from A549 cells. The cells were plated onto 6-well plates. When the culture reached 80% confluence, the culture medium of the A549 cell was removed and the treatments were added. After 24 h of retinol treatment, to obtain SMPs, A549 cells were homogenized in 230 mM mannitol, 70 mM of sucrose, 10 mM of Tris–HCl and 1 mM of ethylenediaminetetraacetic acid (EDTA; pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution also was washed (twice) with 140 mM of KCl and 20 mM of Tris–HCl (pH 7.4) to ensure Mn–SOD release from mitochondria. To quantify $O_2^{-\bullet}$ production, SMP was incubated in a reaction medium consisting of 230 mM of mannitol, 70 mM of sucrose, 10 mM of HEPES-KOH (pH 7.4), 4.2 mM of succinate, 0.5 mM of KH_2PO_4, 0.1 mM of catalase and 1 mM of epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C [43].

2.12. Role of NADPH oxidase in superoxide anion radical production

Lucigenin-dependent chemiluminescence was used to detect $O_2^{-\bullet}$ production, at 37 °C with a Perkin Elmer Microbeta Reader. Briefly, A549 cells were plated onto 96-well plates. When cells reached 90% of confluence the culture medium was exchanged to 100 μL Phosphate-Buffered Saline (PBS, pH 7.4), with or without retinol. Fifteen minutes later 50 μL of 100 μM lucigenin solution was added [44]. To analyze the role of NADPH oxidase in $O_2^{-\bullet}$ production, A549 cells were exposed to NADPH oxidase inhibitors, DPI (100 μM and 1 mM) or apocynin (100 μM and 1 mM). The peak of the chemiluminescence produced (counts/min/10 mm^2) was used for comparisons between groups and for calculating the absolute amount of $O_2^{-\bullet}$ per minute compared with a control. Results were expressed as nmol $O_2^{-\bullet}$/min/mg protein. Protein was measured according to the method of Bradford using bovine serum albumin as standard. All determinations were done in triplicate.

2.13. Confocal immunofluorescence microscopy analysis

A549 cells were washed with phosphate-buffered saline and fixed on chamber slides with 4% paraformaldehyde (PFA) in 4 °C for 15 min. Samples were sequentially treated with 0.15% Triton X-100 (permeabilization) for 10 min and 10% serum (to avoid nonspecific binding) for 30 min. Subsequently, cells were incubated with either of one specific primary antibodies: rabbit polyclonal anti-RAGE (Abcam Inc., ab36647; 1:2000) and rabbit polyclonal anti-NF-κB p65 (C-20) (Santa Cruz Biotechnology Inc., sc-372; 1:400). Fluorescent-labeling was performed by incubating samples with Alexa Fluor 488 goat anti-rabbit (Molecular Probes, A11008, 1:400). For nuclear staining, Prolong Gold Antifade Reagent with DAPI (Molecular Probes, P36931) was used. Images were taken with an Olympus FluoView™ 1000 confocal microscope and subsequently analyzed by using Olympus Fluoview FV1000 Software, ver.3.0. Thresholds discriminating between signal and background were selected by utilizing cells that were only stained with secondary antibodies (negative control).

2.14. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells with the RNAqueous kit (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was synthesized with the Super Script III Reverse Transcriptase kit (Invitrogen, USA) using 0.5 μg of total RNA. Quantitative RT-PCR reactions were performed in triplicate for each sample using the Step One Plus thermocycler (Applied-Biosystems, USA) and the Power SYBR Green PCR kit (Applied-Biosystems, USA). The reactions were carried out in 25 μL following manufacturer's instructions, under appropriate

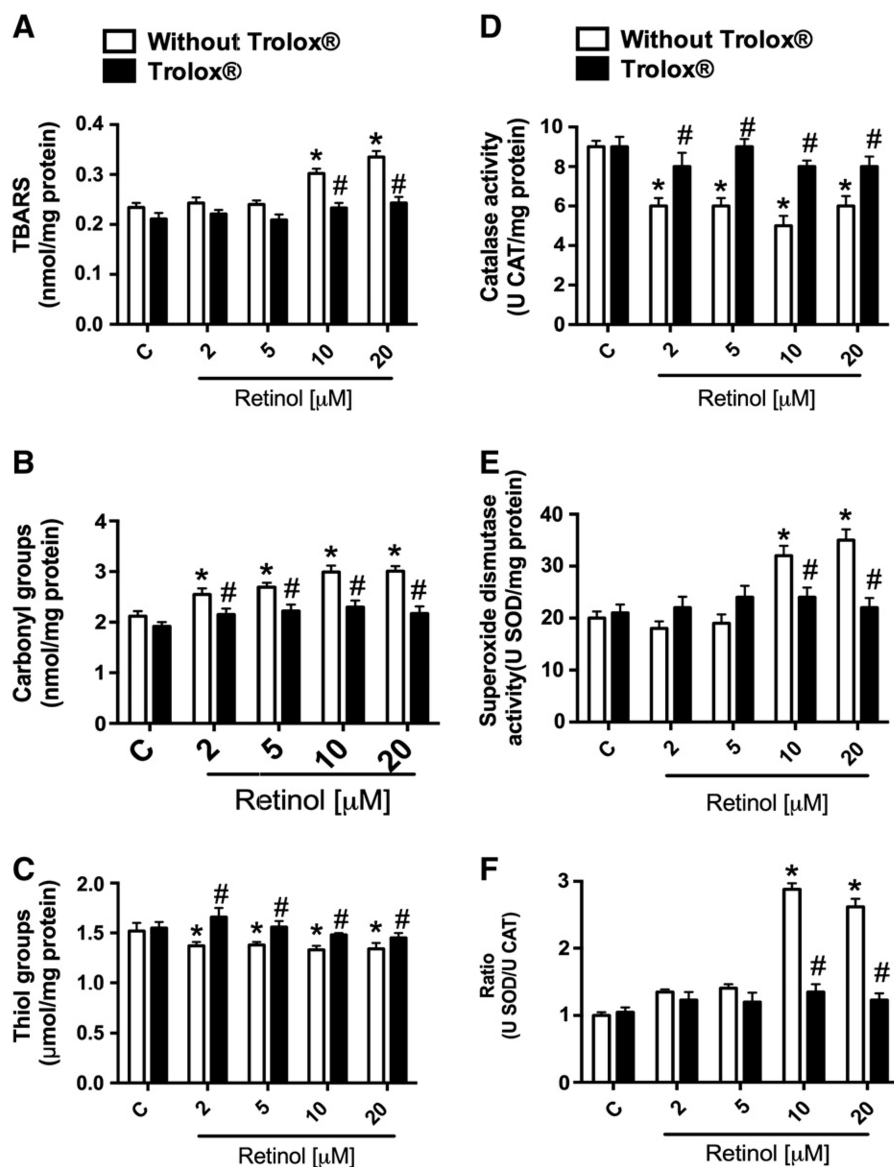


Fig. 1. Parameters of oxidative stress in A549 cells treated with retinol for 24 h. A549 cells were treated with retinol at 2, 5, 10 and 20 μM in the absence or presence of the antioxidant Trolox® at 100 μM. (A) Thiobarbituric acid reactive species (TBARS) levels were assessed as an index for cellular lipid peroxidation; levels of (B) carbonyl were quantified in order to evaluate cell protein oxidative damage and (C) thiol levels were assessed to verify protein redox modification. The activities of the antioxidant enzymes (D) catalase (CAT) and (E) superoxide dismutase (SOD) were also evaluated, and the relative ratio (F) between their activities (SOD/CAT) was calculated. Control group is represented in all graphs by the letter "C". Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group, while #$p<0.05$ vs respective group without Trolox®.

conditions for each primer pair. Quantification was performed using the 2 −ΔΔCT method. The constitutive gene used was GAPDH.

2.15. Cellular fractionation

For nuclear extracts preparation cells were plated onto 6-well plates. When cells reached 80% confluence, the culture medium was removed and treatments were added. To obtain the cytoplasmic extracts and nuclear extracts the cells were washed with cold phosphate-buffered saline and suspended in 0.4 mL hypotonic lysis buffer (10 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L dithiothreitol plus protease inhibitor cocktail) for 15 min. Cells were then lysed with 12.5 μL 10% Nonidet P-40. The homogenate was centrifuged (13,000 ×g, 30 s), and supernatants containing the cytoplasmic extracts (fraction 1) were stored at −80 °C. The nuclear pellet was resuspended in 100 μL ice-cold hypertonic extraction buffer (10 mmol/L HEPES pH 7.9, 0.42 M NaCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol plus protease inhibitors). After 40 min of intermittent mixing, extracts were centrifuged (13,000 ×g, 10 min, 4 °C), and supernatants containing nuclear proteins were collected.

2.16. NF-kB-p65 ELISA assay for the determination of NF-kB activity

A total of 10 μg of nuclear protein extracts was used to determine NF-kB activation (NF-kB p65 ELISA kit, Stressgen/Assays designs) according the manufacturer protocols. This ELISA-based chemiluminescent detection method rapidly detects activated NF-kB complex binding (p65 detection) to a plate-adhered NF-kB consensus oligonucleotide sequence. Kit-provided nuclear extracts prepared from TNF-α stimulated Hela cells were used as a positive control for NF-kB activation. To demonstrate assay specificity, a 50-fold excess of an NF-kB consensus oligonucleotide was used as competitor to block NF-kB binding (data not shown). In addition, a mutated consensus NF-kB oligonucleotide (which do not binds NF-kB) is provided for the determination of binding reactions' specificity (data not shown).

2.17. Electrophoretic-mobility shift assay (EMSA)

To determine NF-kB DNA-binding activity, we performed an electrophoretic mobility shift assay (EMSA) using the biotin-3′end-labeled NF-kB DNA oligonucleotide consensus sequences: 5′CGACACCCCTCGG GAATTCCC-CCACTGGGCC-3′, 3′GCTGTGGGGAGCCCT-TAAGGGGGTGAC CCGG-5′. Briefly, the DNA oligonucleotide consensus sequences were

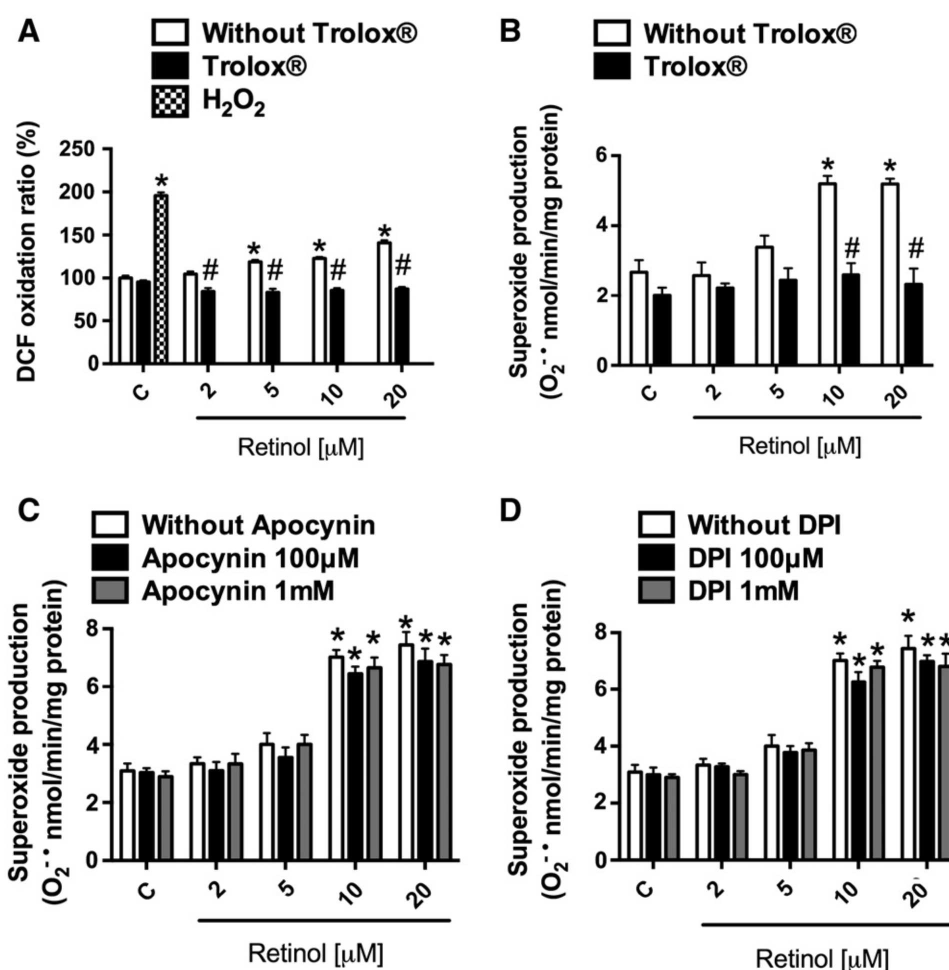


Fig. 2. Intracellular reactive species production by A549 cells subjected to retinol treatment. (A) Cells were treated with different concentrations of retinol in the absence or presence of Trolox® 100 μM for 1 h and the total production of reactive species by living cells was evaluated by the real time DCFH-oxidation assay; H_2O_2 300 μM was used as a positive control for reactive species production and fluorescence intensity was calculated relative to control cells. (B) Superoxide production by submitochondrial particles (SMP) isolated from A549 cells; cells were treated for 24 h with retinol in the absence or presence of Trolox® 100 μM and then SMP were isolated as described in "Materials and methods" and the production of superoxide was assessed by auto-oxidation of adrenaline to adrenochrome. Superoxide production by A549 cells was also evaluated in intact cells by the lucigenin-assay using the NADPH-oxidase inhibitors; different concentrations of (C) apocynin and (D) diphenyliodonium chloride (DPI) were used in order to evaluate the role of NADPH oxidase in retinol-induced superoxide production. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group, while #$p<0.05$ vs respective group without Trolox®.

labeled with biotin-ddUTP in accordance with manufacturer instructions (LightShift Chemiluminescent EMSA kit, Pierce, Rockford, IL, USA). In binding reactions, 20 μL of reaction mixture comprising 15 mM HEPES (pH 7.9), 1 mM dithiothreitol, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $MgCl_2$, 2.5% glycerol and 50 ng/μL poly(dI-dC), 5 μg of nuclear protein extracts, and 30 fmol of biotin-3′end-labeled DNA probes were incubated for 30 min in ice bath. Nucleo-protein complexes were loaded onto the pre-electrophoresis 5.5% non-denaturing polyacrylamide gels in 0.5× Tris–boric acid–EDTA buffer and run at 120 V. The electrophoresed binding reactions were electrotransferred (100 mA for 3 h) in 0.5× Tris–boric acid–EDTA buffer to a nylon membrane positively charged in ice-cold bath. The biotin-ddUTP 3′end-labeled DNA probe was cross-linked with ultraviolet-C (UVC) exposure for 15 min and detected using streptavidin–horseradish peroxidase conjugated. The membranes were exposed to X-ray films for 1–5 min to obtain the adequate signal.

2.18. siRNA knockdown

The Silencer® Select Validated *si*RNA assays (*si*RNA ID# s11914; *si*RNA ID# s11157; *si*RNA ID# s3585; *si*RNA ID# s1166; Ambion® Inc.) were transfected using the *si*PORT™ *Neo*FX™ Transfection Agent (Ambion®, Applied Biosystems Inc.) in agreement with manufacturer's protocol. A549 cells were transfected with 150 nM of siRNAs by reverse transfection and then incubated for 24 h in Opti-MEM to allow knockdown of the proteins, which were confirmed by qRT-PCR. Silencer® Select Negative Control #1 *si*RNA containing scrambled sequences was used as negative control (see supplementary material).

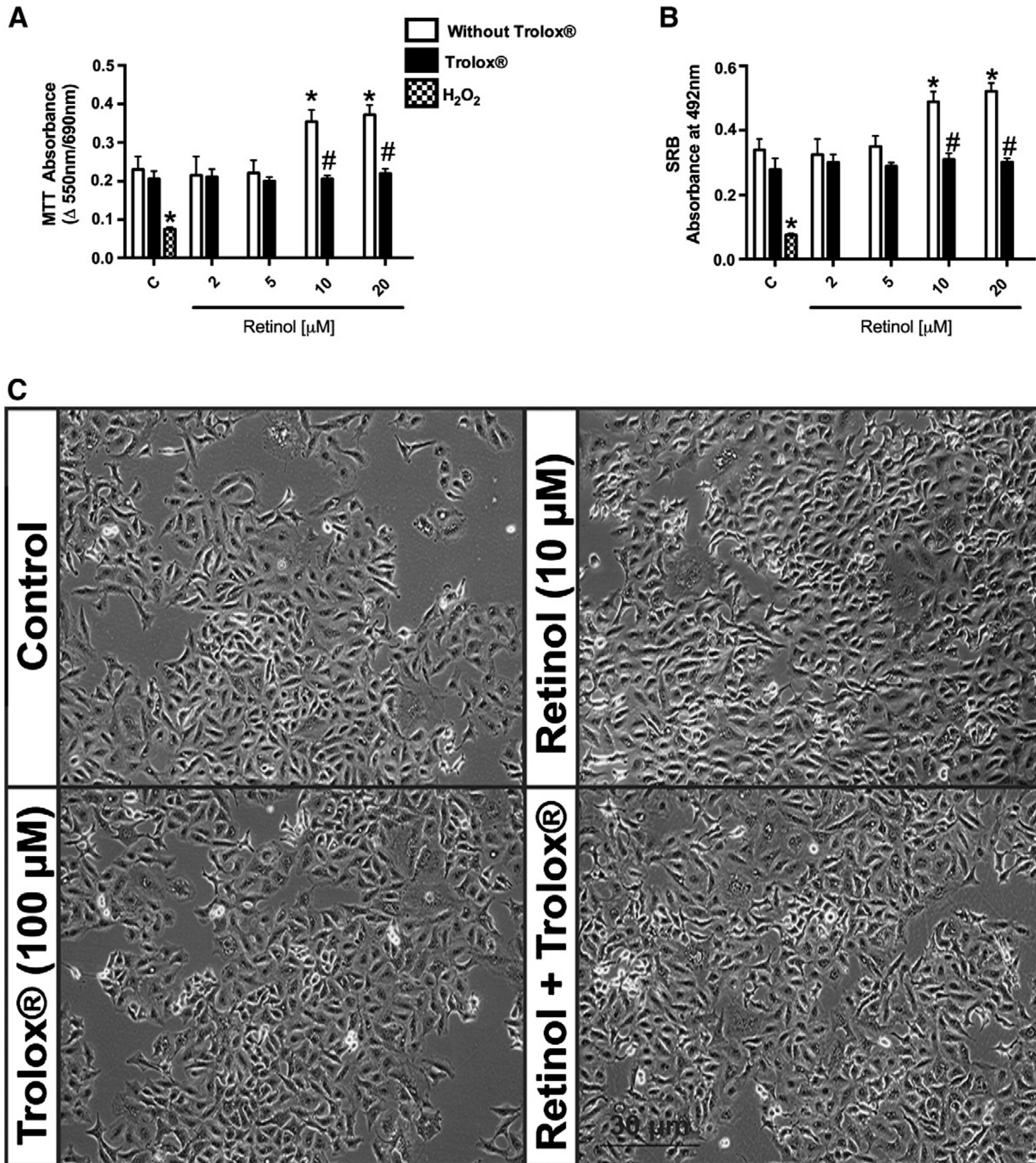


Fig. 3. Cell viability parameters. A49 cells were treated with retinol at 2, 5, 10 and 20 μM in the absence or presence of Trolox® 100 μM for 24 h and different assays were performed to evaluate cell viability after incubation; H_2O_2 (300 μM) was used as a positive control for loss of viability. (A) MTT reduction assay and (B) SRB-incorporation assay. (C) Phase-contrast microscopy of cells treated with retinol 10 μM and Trolox® 100 μM for 24 h. Representative images from three independent observations are shown; data are mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group, #$p<0.05$ vs respective group without Trolox®.

2.19. Immunoblot

To perform immunoblot experiments, A549 cells 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 protein (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes. Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was 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. Membranes were incubated overnight at 4 °C with antibodies, in the presence of 5% skin milk and then washed with TTBS. Anti-rabbit IgG peroxidase-linked secondary antibody was incubated with the membranes for additional 1 h (1:5000 dilution range), washed again and the immunoreactivity was detected by enhanced chemiluminescence using ECL Plus kit. Densitometric analysis of the films was performed with Image J software. Blots were developed to be linear in the range used for densitometry.

2.20. Statistical analysis

Results are expressed as mean values ± standard error of the mean (SEM); p values were considered significant when $p<0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post-hoc Tukey's test whenever necessary.

3. Results

We first analyzed parameters of oxidative stress in cells treated with retinol at the concentrations of 2 μM, 5 μM, 10 μM, or 20 μM for 24 h. We observed increased lipid peroxidation levels in A549 cells that received retinol treatment at 10 μM and 20 μM ($p<.05$) (Fig. 1A). Besides, all doses of retinol increased protein carbonylation levels (Fig. 1B) and decreased protein thiol content (Fig. 1C) in relation to control group, confirming that retinol treatment exerted a pro-oxidant effect in A549 cellular proteins. Retinol treatment also modified the activity of antioxidant enzymes. We observed a decrease in CAT activity with retinol treatment at 5 μM, 10 μM and 20 μM (Fig. 1D) while cells treated with retinol at 10 μM and 20 μM had increased SOD activity ($p<.05$, Fig. 1E). These modifications resulted in a significant imbalance of the SOD/CAT ratio (Fig. 1F), which results in increased cellular free radical production. The effects caused by retinol treatment on redox parameters were inhibited by co-treatment with the antioxidant Trolox® at the concentration of 100 μM. Trolox® is a hydrophilic analog of α-tocopherol. This indicates that the effects of retinol on parameters of oxidative stress observed here were indeed mediated by an increase in free radical production.

To confirm that retinol enhanced cellular ROS/RNS production, we evaluated the production of ROS/RNS by A549 cells by the real-time DCFH oxidation assay. Retinol increased the rate of intracellular reactive species production in a dose-dependent manner ($p<.05$), reaching a maximum effect at 20 μM (Fig. 2A). The co-treatment with Trolox® reversed the increase in DCFH oxidation induced by retinol, confirming that this effect was mediated by increased ROS/RNS production; H_2O_2 300 μM was used as a positive control for DCF fluorescence. Since mitochondria are major cellular sites of ROS production due to oxidative metabolic activity, we decided also to evaluate mitochondrial ROS production in submitochondrial particles (SMP) isolated from retinol-treated cells. Retinol at 5, 10, and 20 μM significantly increased mitochondrial $O_2^{-\bullet}$ production (Fig. 2B). Additionally, we analyzed the involvement of another source of cellular ROS, the enzyme NADPH oxidase, in $O_2^{-\bullet}$ production by intact cells. By real-time lucigenin assay, we observed that retinol (10 and 20 μM) increased $O_2^{-\bullet}$ production (Fig. 2C and D). When A549 cells were co-treated with the inhibitors of NADPH-oxidase apocynin (Fig. 2C) and diphenylene iodonium (DPI, Fig. 2D), $O_2^{-\bullet}$ production was not altered. Altogether, these results indicate that the increase in $O_2^{-\bullet}$ production induced by retinol is mainly due to mitochondrial activity and is not mediated by NADPH-oxidase activation.

ROS/RNS excessive production is associated with disruption of cell cycle regulatory mechanisms [45]. ROS/RNS confer a growth advantage to tumor cells by facilitating mitogenic signaling through

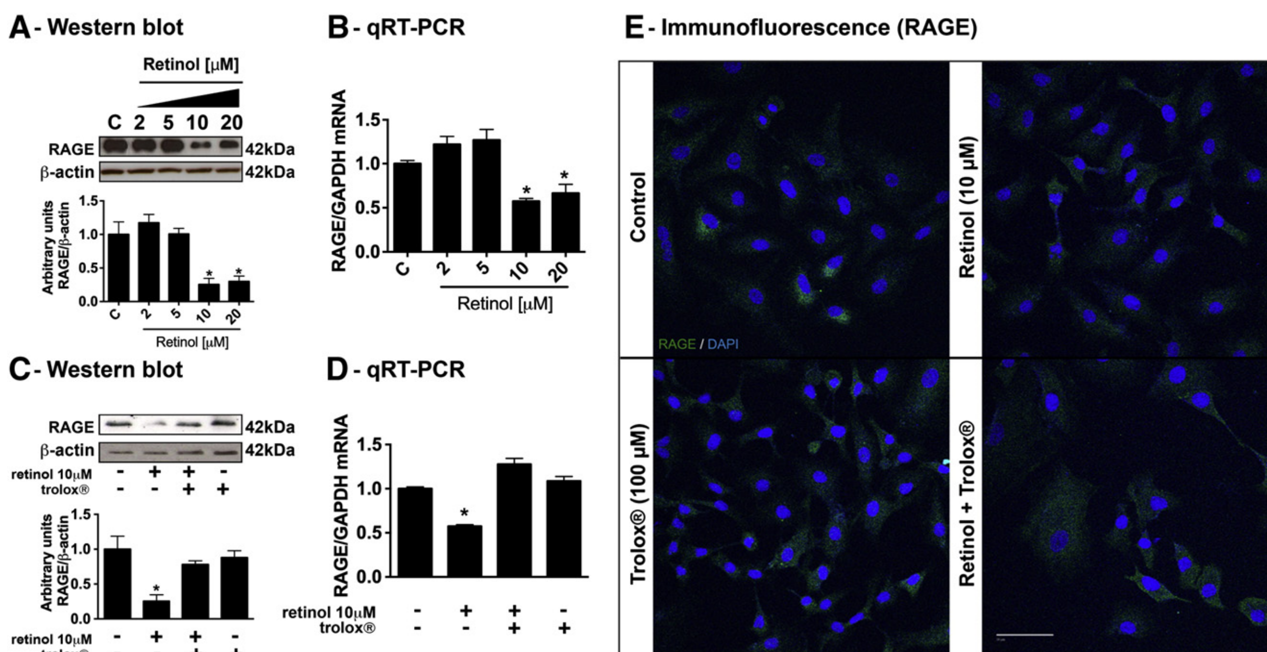


Fig. 4. RAGE expression in cells treated with retinol for 24 h. A549 cells were treated with retinol at 2, 5, 10 and 20 μM and total protein homogenates were separated by SDS-PAGE in order to evaluate the immunocontent of RAGE by (A) western blot; β-actin immunocontent was used as internal control. Total RNA of cells treated with retinol was extracted and RAGE expression was also evaluated by (B) quantitative RT-PCR (qRT-PCR) as described in "Materials and methods" using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control for constitutive expression. (C) The effect of the antioxidant Trolox® (100 μM) on the modulation of RAGE immunocontent by retinol 10 μM was also evaluated by western blot; (D) qRT-PCR analysis was also performed for the same experimental conditions. RAGE immunocontent in cells treated with retinol 10 μM in the absence or presence of Trolox® was also evaluated by (E) confocal immunofluorescence microscopy. Representative images are shown. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

the activation of several stress kinase pathways as MAPKs [46]. ROS/RNS may also play a role on the effects of NF-kB regulation of cellular proliferation or differentiation through involvement of cyclin D1 [47]. Moreover in cancer cells ROS/RNS is involved in pathways that activate the hypoxia-inducible factor α (HIF-1α)[48]. As a consequence, oxidative stress often induces apoptosis, proliferation or cell death. As our results indicate that retinol affected the cell redox homeostasis, we analyzed A549 cell viability after retinol treatment for 24 h. Retinol did not cause any loss in cell viability, as indicated by MTT and SRB-based assays (Fig. 3A and B). On the other hand, at 10 and 20 μM, retinol significantly enhanced MTT reduction and SRB incorporation, indicating an increase in the number of viable cells ($p<0.05$). This increase was confirmed in phase-contrast microscopy imaging (Fig. 3C). Co-treatment with Trolox® also blocked these effects.

In lungs, RAGE expression is considered essential for adult pulmonary homeostasis [31] and its down-regulation is progressively associated to higher tumor (TNM) stages [33]. Given that RAGE is associated with differentiation, cell polarity and organization of the epithelium, its decrease in lung tumors might contribute to the loss of polarization and differentiation. This assumption is strongly supported by observation that RAGE contributes to cell growth as an epithelial layer on collagen, a major compound of the epithelial basement membrane [32]. Recently was identified that RAGE is at least one of the critical receptors for chondroitin sulfate and heparan sulfate. These sulfated glycosaminoglycans are expressed at the tumor cell surface and involved in experimental lung metastasis [49]. In previous works, we observed that retinol regulates RAGE expression through ROS-dependent mechanisms in Sertoli cells [50]. Since retinol supplementation was reported to increase lung cancer incidence in risk populations [25] and given the widely reported association of this type of cancer with oxidative stress [51], we decided to investigate the effect of retinol on RAGE expression in A549 cells.

Retinol at 10 and 20 μM decreased the immunocontent of RAGE, as observed by western blot analysis (Fig. 4A, $p<.05$) and immunofluorescence assays (Fig. 4E); besides, RAGE mRNA expression was also

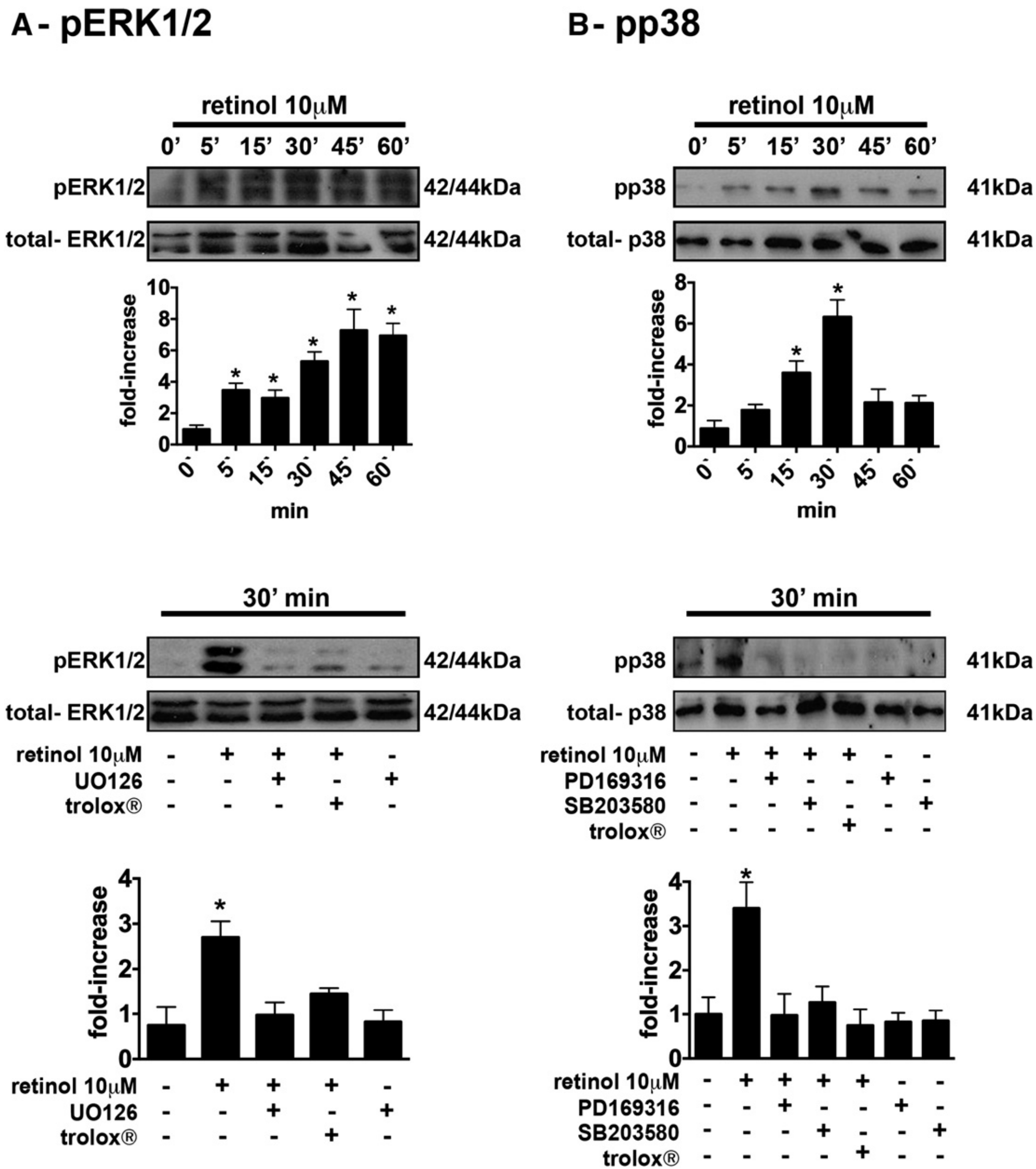


Fig. 5. Effect of retinol on the phosphorylation of (A) ERK1/2 and (B) p38. A549 cells were treated with retinol 10 μM up to 60 min and the phosphorylation state of ERK1/2 and p38 was analyzed in total cell homogenates by western blot. Graphs show the relative quantification of phosphorylated isoforms of ERK1/2 and p38 in relation to their total immunocontent. The effect of the antioxidant Trolox® in cells treated with retinol 10 μM for 30 min on ERK1/2 and p38 phosphorylation was compared with the effect of co-treatment with the specific pharmacologic inhibitors UO126 10 μM (ERK1/2 inhibitor), PD169616 10 μM and SB203580 10 μM (p38 inhibitors). Representative images (western blots) are shown. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

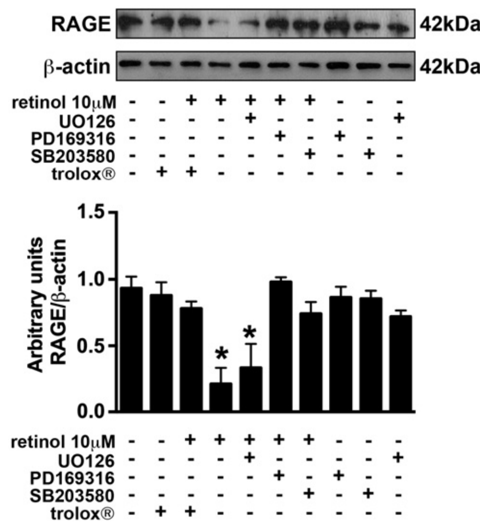
downregulated by retinol treatment, as observed in the qRT-PCR assay (Fig. 4B). To evaluate the mechanisms involved in RAGE down-regulation, we used retinol at 10 μM in further experiments. To establish if the mechanism of RAGE downregulation was related to the increase in ROS/RNS caused by retinol, we evaluated RAGE expression in cells co-treated with Trolox®. We observed that the antioxidant co-treatment inhibited the effect of retinol on RAGE downregulation (Fig. 4C, D and E). Taken together, these data indicate that retinol down-regulates RAGE expression by a mechanism dependent on ROS/RNS production.

To further explore the mechanism by which retinol downregulates RAGE expression, we decided to search for possible cellular targets of oxidative stress which could affect signaling pathways regulating RAGE expression. Mitogen-activated protein kinases (MAPKs) are a family of proteins involved in a wide range of cell responses mediated by reactive species and oxidative stress [52], and previous works observed that retinol is able to induce MAPK activation by an oxidant-mediated mechanism [12,53,54]. Thus, we analyzed the effect of retinol on the activation state of the MAPKs ERK1/2, JNK and p38. MAPK phosphorylation is generally triggered within few minutes after cell stimulation, so we incubated A549 cells with retinol for increasing periods up to 60 min and performed immunoblots to detect the phosphorylated (i.e. active) forms of these protein kinases. Retinol stimulated ERK1/2 and p38 phosphorylation during the 60 min incubation (Fig. 5A and B), but no effect was observed on JNK phosphorylation (data not shown). ERK1/2 phosphorylation steadily increased with time, while p38 activation peaked at 30 min of incubation and then declined. Trolox® co-treatment inhibited the retinol-induced phosphorylation of both ERK1/2 and p38, indicating that the effect of retinol on these kinases was mediated by ROS/RNS.

We next wanted to establish whether RAGE downregulation by retinol was mediated by ERK1/2 and p38. For this purpose, A549 cells were incubated with pharmacological inhibitors of these protein kinases prior to retinol treatment and RAGE expression was evaluated by immunoblot and qRT-PCR (Fig. 6). The ERK1/2 inhibitor UO126 (10 μM) had no effect on RAGE protein (Fig. 6A) and mRNA (Fig. 6B) downregulation by retinol; on the other hand, the p38 inhibitors PD169316 (10 μM) and SB203580 (10 μM) inhibited the effect of retinol on RAGE (Fig. 6A and B). Since this result indicated p38 as the main mediator of retinol on RAGE regulation, we transfected A549 cells with specific siRNA to block p38-α (MAPK14) and p38-β (MAPK11) isoforms in order to confirm the involvement of p38 in the modulation of RAGE expression. Silencing of either p38-β (Fig. 7A and B) or p38-α (Fig. 7C and D) isoforms resulted in blockade of RAGE protein and mRNA downregulation by retinol. This result was also confirmed by confocal microscopy visualization of RAGE immunostaining (Fig. 8). These results, altogether, indicate that p38 activation, in response to retinol-induced ROS/RNS production, is a necessary step in the signal pathway of RAGE downregulation in A549 lung cancer cells.

The activity of several different transcription factors is regulated by p38-controlled signal pathways. Since NF-kB is widely described as a major modulator of RAGE expression in different cell types, including in lungs [55], we decided to investigate the involvement of this transcription factor, along with p38 and ROS/RNS, in the mechanism of RAGE downregulation by retinol. A549 cells were treated with retinol for 24 h and the cytosolic and nuclear fractions were isolated to perform western blot analysis for p65. We observed that retinol treatment decreased p65 cytosolic content (Fig. 9A) and increased p65 nuclear content (Fig. 9B) after a 2 h period of incubation, indicating the induction of p65 nuclear translocation, an essential step in NF-kB activation. Trolox® reversed the effect of retinol on p65 nuclear translocation, confirming the involvement of ROS/RNS in this effect. We also isolated nuclear proteins from A549 cells treated with retinol and performed EMSA and DNA-binding ELISA to evaluate NF-kB ligand activity, another step in NF-kB activation. In these assays, retinol also induced an increase in NF-kB binding activity and this was reversed by Trolox® (Fig. 10A and B). Besides, confocal microscopy analysis using an anti-p65 antibody confirmed the induction of p65 nuclear translocation in A549 cells treated with retinol (Fig. 10C). These results indicate that retinol induces the activation of NF-kB in A549 cells by a redox-sensitive mechanism.

Next, we wanted to establish the relationship between p38 and NF-kB; for this purpose we evaluated the effect of p38 inhibition on NF-kB activation. Both p38 inhibitors (PD169316 and SB203580) blocked the decrease in cytosolic p65 immunocontent (Fig. 11A) and the increase in nuclear p65 immunocontent (Fig. 11B) induced by retinol, which indicate that nuclear translocation of NF-kB is mediated by p38 activation. Besides, NF-kB ligand activity was inhibited by pharmacological inhibition of p38 (Fig. 11C). The effect of the p38 inhibitors was similar to the inhibitory effect of the antioxidant Trolox® on



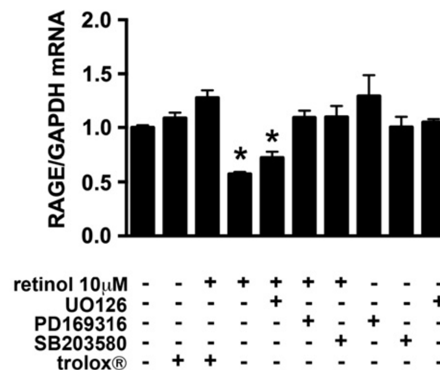


Fig. 6. Effect of ERK1/2 and p38 inhibitors on RAGE downregulation by retinol. A549 cells were incubated for 24 h with retinol 10 μM in the absence or presence of UO126 10 μM (ERK1/2 inhibitor), PD169616 10 μM and SB203580 10 μM (p38 inhibitors) or Trolox® 100 μM. Total protein homogenates were used for (A) western blot analysis of RAGE immunocontent and total RNA was extracted for (B) qRT-PCR analysis. β-Actin immunocontent was used for western blot internal control and GAPDH was used for qRT-PCR internal control of constitutive expression. Representative images (western blots) are shown. Data represent mean values ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, * $p<0.05$ vs the control group.

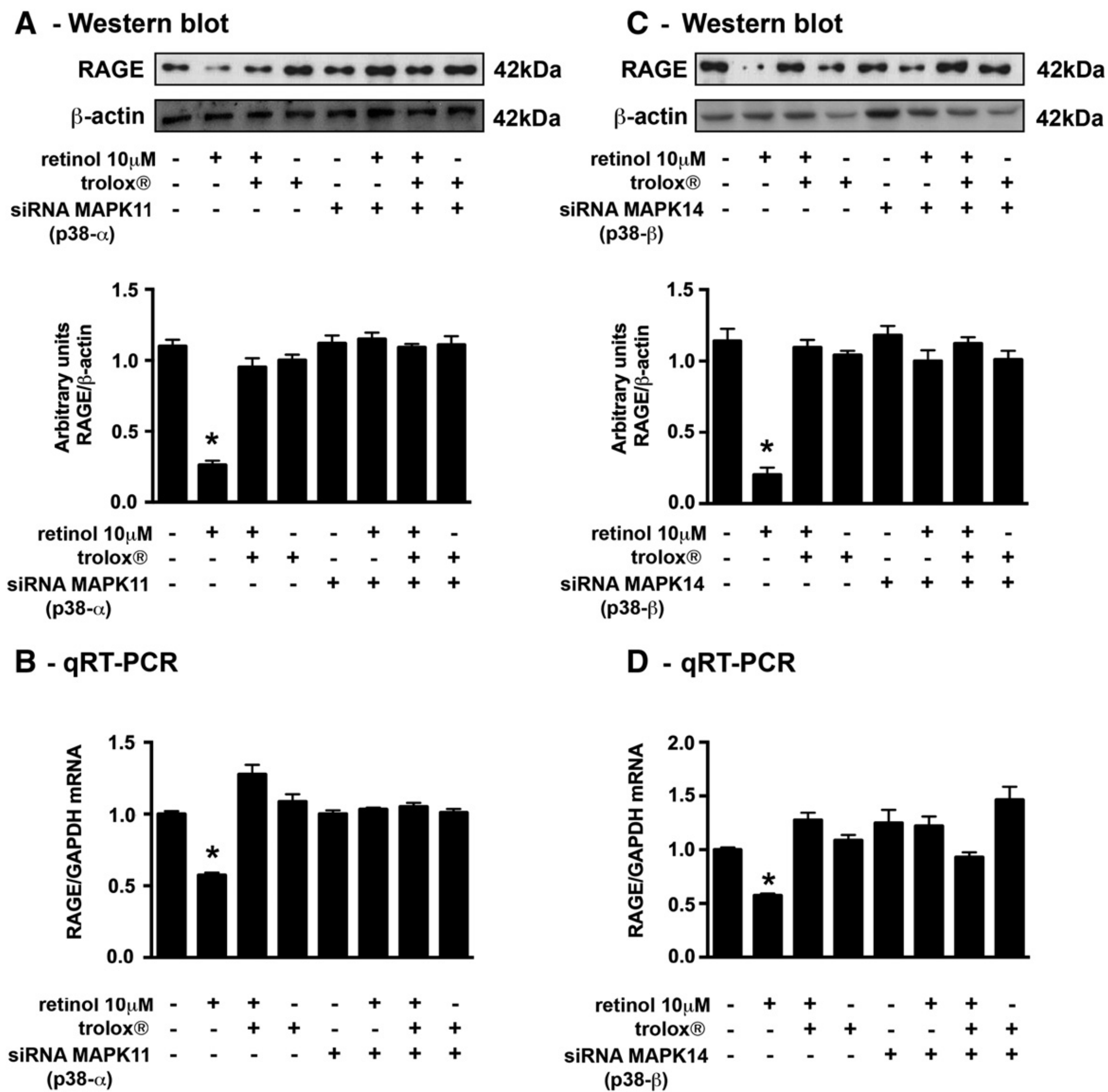


Fig. 7. RAGE expression in p38-silenced A549 cells. Cells were transfected with siRNA sequences for either MAPK11 (p38-β) or MAPK14 (p38-α) and subjected to retinol 10 μM treatment with or without Trolox® 100 μM. The effect of retinol on RAGE expression was evaluated by western blot and qRT-PCR. In silenced p38-β cells, (A) western blot analysis of RAGE immunocontent and (B) qRT-PCR for RAGE expression demonstrated that p38 silencing inhibits RAGE downregulation by retinol. Similar results were observed in p38-α silenced cells, as indicated by (C) western blot and (D) qRT-PCR analysis. Representative images (western blots) are shown. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

NF-kB activation. These results, altogether, indicate that p38 activation is a necessary, upstream step in NF-kB activation by retinol.

As the last step, we evaluated the effect of NF-kB inhibition on RAGE expression. A549 cells were treated with retinol in the presence of the cell-permeable peptide SN50 100 μg/mL, an inhibitor of NF-kB, and RAGE expression was evaluated. We observed that SN50 reversed the effect of retinol on RAGE protein (Fig. 12A) and mRNA expression (Fig. 12B). The extent of the effect of SBN50 on RAGE was similar to the effect of the p38 inhibitors PD169316 and SB203580 on RAGE. Also, the effect of SN50 was similar to the effect of Trolox®. To confirm the involvement of NF-kB on RAGE modulation by retinol, A549 cells were transfected with siRNA for p65 and RAGE expression was evaluated. In silenced-p65 cells, RAGE downregulation by retinol was inhibited in an effect similar to the antioxidant Trolox®, as observed by western blot (Fig. 13A), qRT-PCR (Fig. 13B) and confocal immunofluorescence analysis (Fig. 13C). Taken together, the results presented here suggest that retinol, by increasing ROS/RNS production, induces the p38-dependent activation of NF-kB, which is responsible for the downregulation of RAGE in A549 lung cancer cells (Fig. 14).

4. Discussion

The redox properties of vitamin A and related compounds have been the subject of intense debate in the present days. Initially, retinoids were claimed to exert important antioxidant functions in biological systems, and this belief stimulated the use of retinoids as antioxidants and nutritional supplements for prevention and treatment of diverse diseases [4,6,9]. Free radicals and other forms of reactive species have been correlated with increased risk and incidence of respiratory diseases, such as asthma, chronic obstructive pulmonary disease, parenchymal lung diseases (e.g., idiopathic pulmonary fibrosis and lung granulomatous diseases) and cancer [56–60]. In some cases, serum retinol deficiency was reported in these diseases and retinoid supplementation or pharmacological administration has been proposed as "antioxidant therapies" for treatment and/or prevention of these conditions. Vitamin A therapy (mainly as retinol palmitate) at moderate to high doses (30,000–300,000 IU/day) is prescribed to patients under dermatological disturbances. Besides, vitamin A at doses exceeding 150,000 IU/day is administered to infants, children and young adults

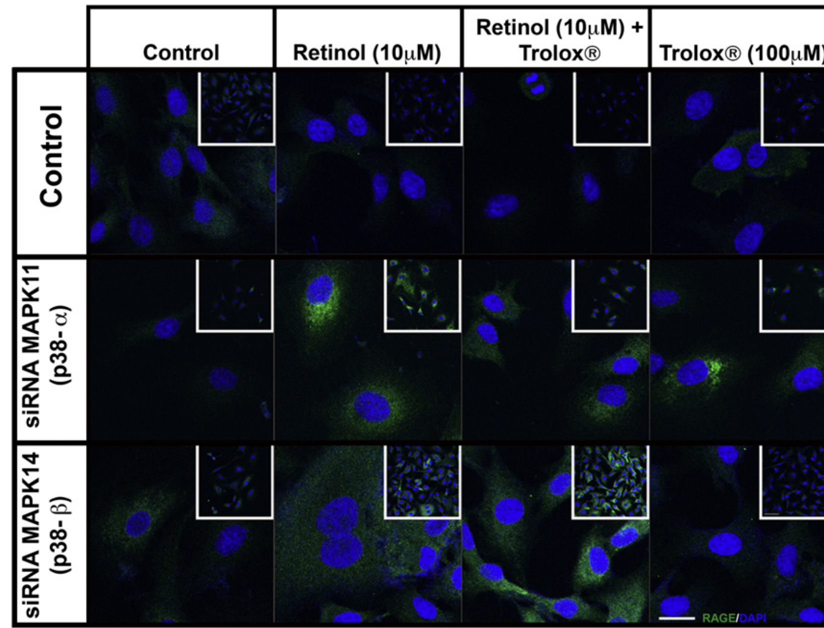
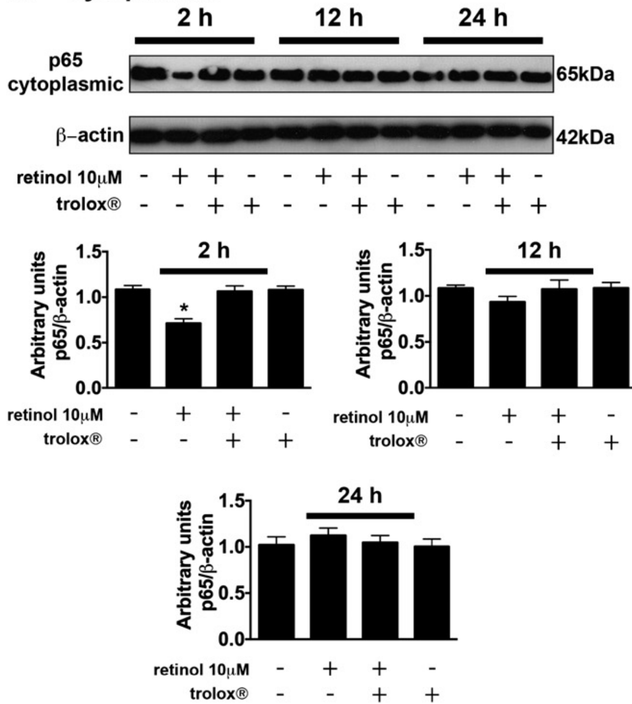


Fig. 8. Confocal immunofluorescence microscopy analysis to evaluate RAGE immunocontent in A549 cells transfected with siRNA sequences for either MAPK11 (p38-β) or MAPK14 (p38-α) and subjected to retinol 10 μM treatment with or without Trolox® 100 μM. Representative images (40×, and 120×) from three independent experiments are shown. Green = RAGE; blue = DAPI.

during leukemia treatment [61]. Clinical trials have been carried out based on the potential antioxidant role of retinoids, but in some studies retinoid supplementations had to be discontinued due to increased mortality related to lung cancer and cardiovascular disease incidence [21,25]. It was observed that in hypervitaminosis A, the levels of plasma and tissue retinol do not correlate with the increased intake [62,63], suggesting that retinol is converted to several other metabolites when increasing doses are ingested. Cell culture as well as other in vitro assays



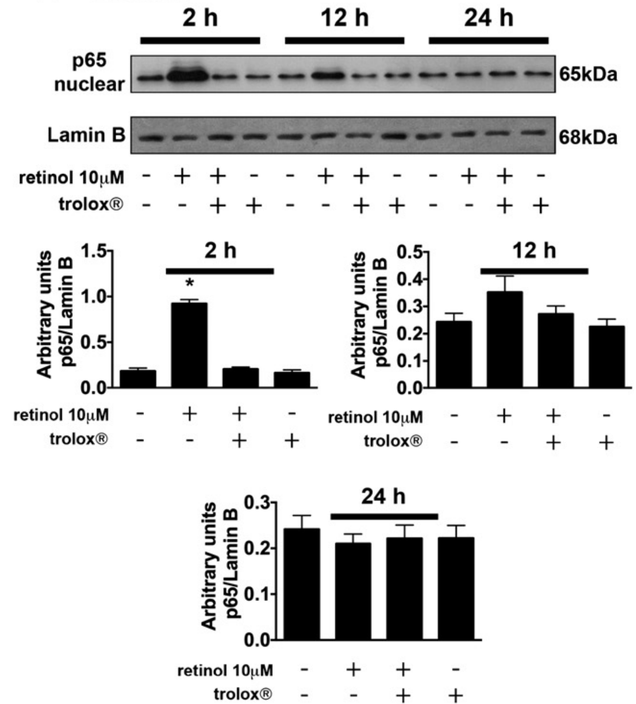


Fig. 9. Translocation of p65 from cytosolic to nuclear fraction in A549 cells treated with of retinol 10 μM with or without Trolox® 100 μM for 2, 12 and 24 h. (A) Immunoblot and quantification of the p65 immunocontent (relative to β-actin) in the cytosolic fraction. (B) Immunoblot and quantification of the p65 immunocontent (relative to lamin B) in the nuclear fraction. Representative images (western blots) are shown. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

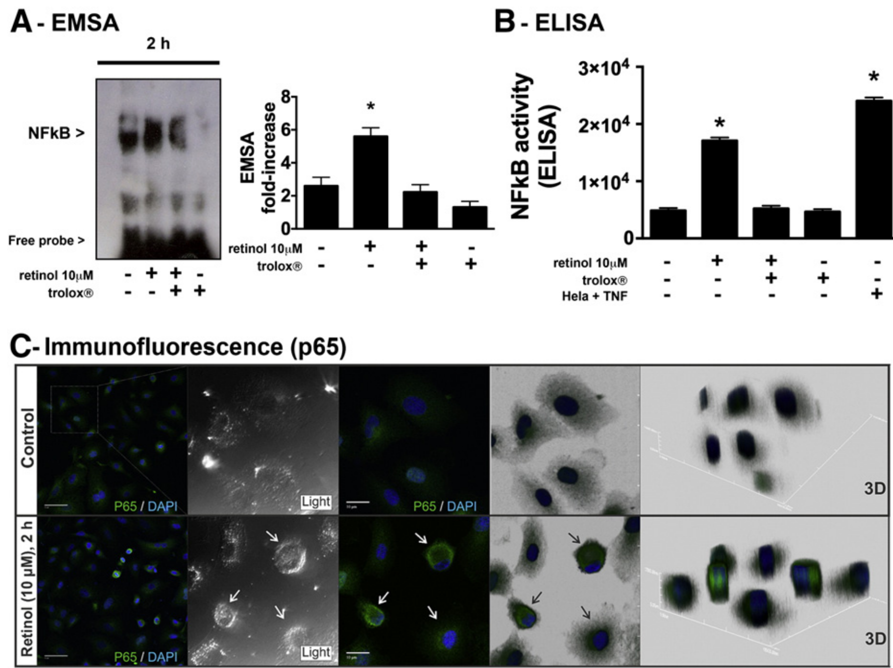


Fig. 10. NF-kB activation by retinol in A549 cells. (A) Electro-mobility shift assay (EMSA); A549 cells were treated with retinol 10 μM in the absence or presence of Trolox® 100 μM for 2 h and the nuclear proteins were isolated as described in "Materials and methods" and subjected to EMSA; a representative run is shown. NF-kB ligand-activity in nuclear proteins isolated from A549 cells treated with retinol 10 μM for 2 h was also evaluated using an (B) ELISA-based assay with plate-adhered oligonucleotides containing an NF-kB consensus binding sequence. (C) Confocal immunofluorescence microscopy analysis with an antibody against p65 shows the translocation of the p65 subunit from the cytosol to the nucleus in cells treated with retinol 10 μM for 2 h. The white arrows indicate the subcellular localization of p65. Representative images from three independent experiments are shown; data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

confirmed that retinoids also presented cytotoxic and/or pro-oxidant effects, causing oxidative damage to biomolecules [10,15]. Additionally, retinol is known to regulate gene transcription by activating cell surface receptors such as STRA6 that signal through activation of JAK/STAT signaling cascades [16]. Thus, the redox properties of vitamin A and other retinoids may vary in biological systems, and the side effects resulting from preventive or therapeutic supplementations should not be neglected when considering the use of such protocols.

We observed that retinol treatment at different doses induced a pro-oxidant state in A549 cells. Retinol increased lipid peroxidation, protein carbonylation, and decreased protein thiol content. Moreover, the activities of CAT and SOD were also modulated by retinol. It is known that retinol auto-oxidation in vitro increases $O_2^{-\bullet}$ production [14]. Furthermore, van Helden et al. showed that both β-carotene and its metabolites (vitamin A, retinal, and retinoic acid) were able to increase hydroxyl radical ($OH^\bullet$) formation in a system containing

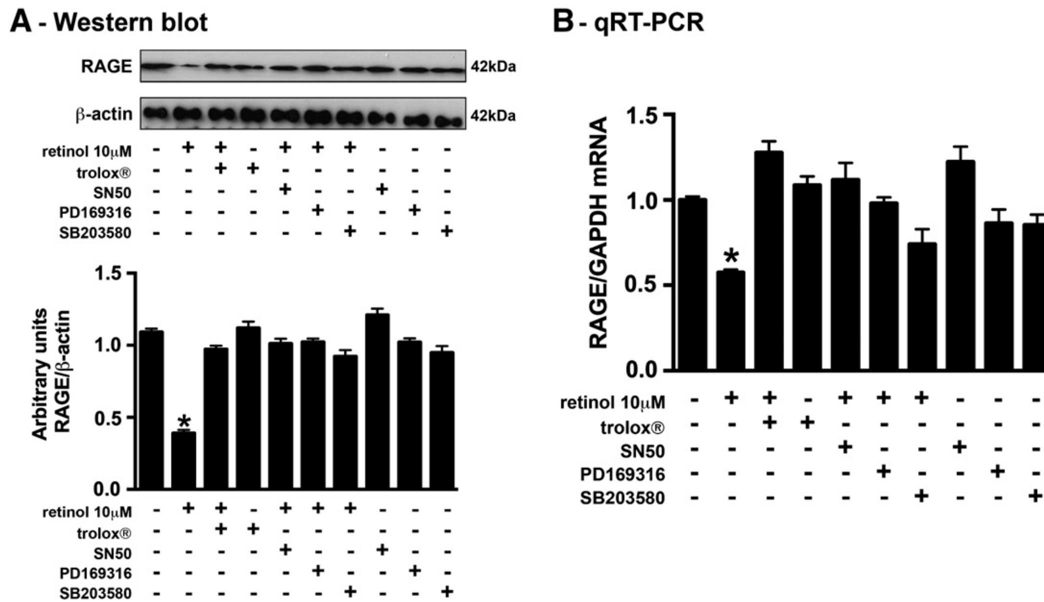
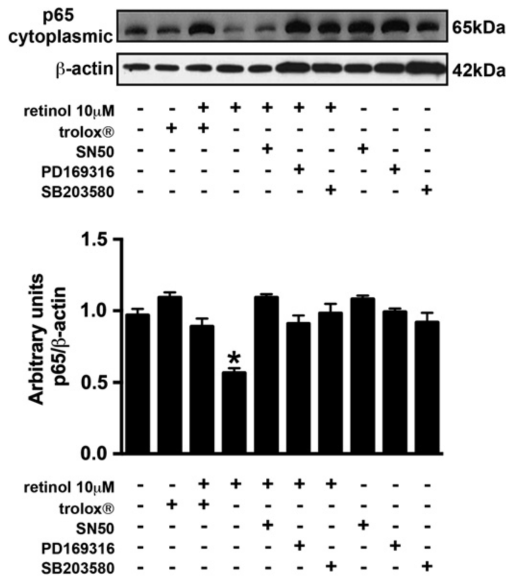


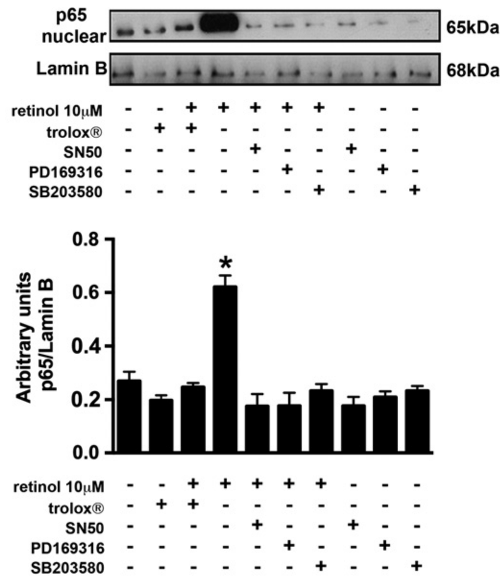
Fig. 11. RAGE expression in A549 cells treated with NF-kB and p38 inhibitors. A549 cells were treated with retinol 10 μM for 24 h and the effect of the NF-kB inhibitor peptide SN50 (10 μg/mL) on RAGE expression was compared with the effect of Trolox® 100 μM and the p38 inhibitors PD169616 (10 μM) and SB203580 (10 μM). (A) Total protein homogenate was used for western blot analysis of RAGE immunocontent relative to the total content of β-actin. (B) Total RNA was extracted and used for qRT-PCR analysis of RAGE expression relative to the GAPDH expression levels. Representative immunoblot is shown; data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *$p<0.05$ vs the control group.

iron and hydrogen peroxide (H_2O_2) through the Fenton reaction [64]. Here, both $O_2^{-\bullet}$ and $OH^\bullet$ seem to be formed at a high rate in A549 cells treated with retinol, and this may be due to modulation of antioxidant enzymes.

Previous studies showed that vitamin A leads to an impairment of electron transfer system, thus increasing the rate of $O_2^{-\bullet}$ production [65,66]. We observed increased SOD activity and $O_2^{-\bullet}$ production during retinol treatment in A549 cells. Moreover, a decrease in CAT activity was observed. It is well described that $O_2^{-\bullet}$ is a potent inhibitor of CAT [67]. Together, these results indicate a possible exacerbation in $O_2^{-\bullet}$ concentration in our experimental model. Increased $O_2^{-\bullet}$ may favor SOD activity because this species is the major SOD allosteric activator. Increased SOD activity and decreased CAT activity occurring simultaneously may result in enhanced formation of H_2O_2. Second, an excess of H_2O_2 facilitates the production of OH, a powerful oxidant species, through Fenton reaction. In addition, H_2O_2 is relatively stable and able to diffuse into the nucleus, where it can react with DNA-associated transition metals to form $OH^\bullet$. Oxidatively damaged DNA is known to be pro-mutagenic and is therefore an important marker for carcinogenesis. An imbalance between SOD and CAT activities (SOD/CAT ratio) is thought to facilitate oxidative-dependent alterations in the cellular environment, thus impaired SOD/CAT ratio is very likely to culminate in increased oxidative damage to biomolecules. In fact, an optimal ratio of CAT and SOD activities varies according to each cell type and tissue. An optimal ratio is the one that balances the detoxification of $O_2^{-\bullet}$ by SOD with detoxification of H_2O_2 by CAT. Many authors have shown that an imbalance between SOD/CAT is responsible for increasing oxidative stress in biological systems [68].

The increase in protein carbonyls as well as in lipid peroxidation observed in A549 cells subjected to retinol treatment may probably be due to imbalance of SOD/CAT and increased $O_2^{-\bullet}$ production. A large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox signaling pathways depends on the redox state of the thiol group present both in proteins and in low molecular-





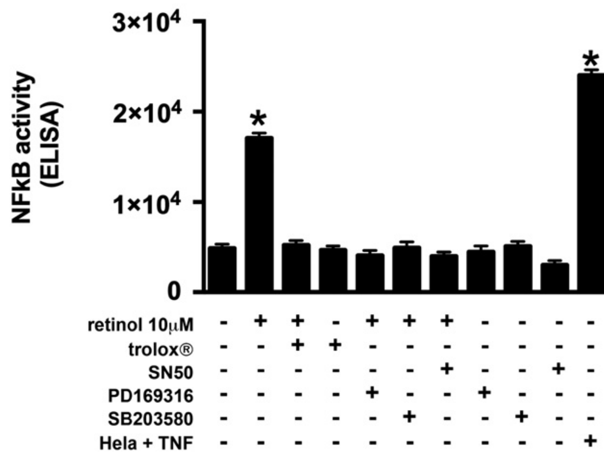


Fig. 12. Inhibition of p65 translocation from cytosolic to nuclear fraction and NF-kB binding activity by pharmacologic inhibitors of NF-kB and p38 in A549 cells treated with retinol. A549 cells were treated with retinol 10 μM in the absence or presence of Trolox® (100 μM), SN50 (10 μg/mL), PD169616 (10 μM) and SB203580 (10 μM) for 2 h; cytosolic (A) and nuclear (B) fractions were separated and subjected to western blot analysis of p65 immunocontent, using β-actin as internal control in the cytosolic fraction and lamin B as internal control in the nuclear fraction. (C) Nuclear proteins were isolated as described in "Materials and methods" and subjected to a ELISA-based assay with plate-adhered oligonucleotides containing an NF-kB consensus binding sequence. Representative immunoblots are shown; data are mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, $^*p<0.05$ vs the control group.

weight molecules. Numerous cell signaling proteins are regulated by alterations of protein thiol groups [69]. This signaling regulates cell proliferation, differentiation and death. Increased protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in a widespread cellular dysfunction [56]. Additionally, increased oxidative damage to proteins might result in increased free iron, because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state. The increase in H_2O_2 production and consequent $OH^•$ formation may also be associated with increase in lipid peroxidation. Products of lipid peroxidation are toxic; lipid hydroperoxides can directly inhibit enzymes.

Retinol treatment in A549 cells decreased RAGE levels. This effect was mediated by a mechanism dependent on intracellular reactive species formation and both activation of p38 and NF-kB. We observed that retinol concentrations up to 5 μM did not induce intracellular reactive species formation and also did not affect RAGE levels. Retinol pro-oxidant concentrations (10 μM and 20 μM) downregulated RAGE in A549 cells and the antioxidant co-treatment with Trolox® blocked this effect. Trolox® abolished intracellular reactive species formation by retinol 10 μM and blocked the activation of p38 and ERK1/2, evidencing the involvement of free radicals in MAPK activation by retinol. The association of MAPK signaling pathways with oxidative stress has been demonstrated in numerous reports; transient MAPK activation is associated with cell proliferation, whereas prolonged MAPK activation may promote cell death. Besides, previous works have shown the involvement of MAPK activation in regulatory mechanisms of RAGE expression [70,71]. Here, RAGE downregulation was blocked by p38 inhibition. Retinol treatment induced both ERK1/2 and p38 activation through ROS/RNS production, but only p38 MAPK pharmacological inhibitors were able to block the effect of retinol on RAGE levels; p38 silencing by siRNA transfection produced a similar effect, confirming the involvement of this MAPK in this effect. Taken together, our results strongly indicate that pro-oxidant concentrations of retinol enhance cellular ROS/RNS production and the consequent redox-mediated activation of p38 and ERK1/2; phosphorylated p38, in turn, induces a downstream cascade of events that culminate in RAGE expression downregulation. According to our data, this downstream cascade involves NF-kB activation.

Diverse stimuli including alterations in the intracellular redox state may induce NF-kB activation [72]. NF-kB is a transcription factor

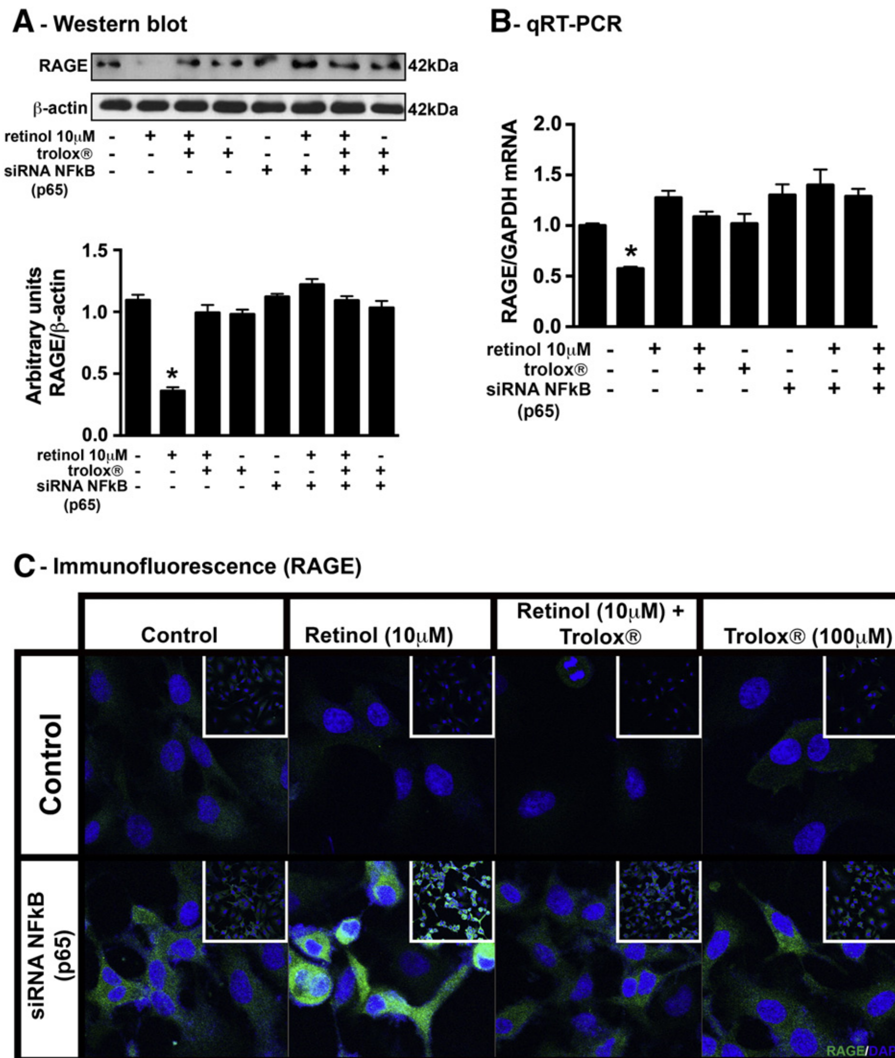


Fig. 13. RAGE expression in silenced-NF-kB cells. A549 cells were transfected with siRNA for NF-kB (p65) and then subjected to retinol (10 μM) treatment with or without Trolox® (100 μM) for 24 h. Total protein homogenates were isolated and used for (A) western blot analysis of RAGE and β-actin immunocontent. (B) Total RNA was extracted and used for qRT-PCR analysis with GAPDH as internal control for constitutive expression. (C) Confocal immunofluorescence microscopy analysis of RAGE immunocontent in A549 cells transfected with p65 siRNA and subjected to the treatments described above; representative images are shown (40×, and 120×). Green = RAGE; blue = DAPI. Data represent mean ± S.E.M from three independent experiments (n = 6 per group). One-way ANOVA followed by the post hoc Tukey's test, *p<0.05 vs the control group.

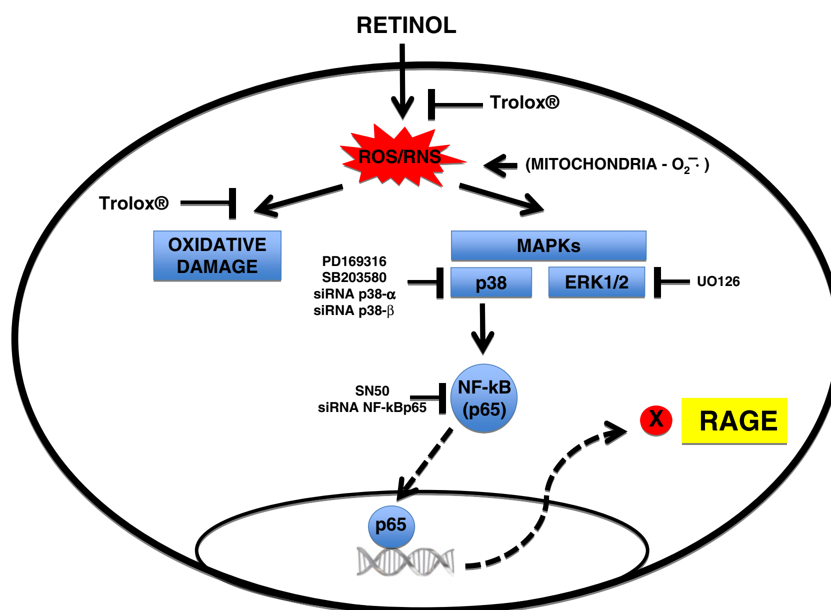


Fig. 14. Schematic overview of the signal cascade triggered by retinol in A549 lung cancer cells. Retinol enhances reactive oxygen/nitrogen species (ROS/RNS) production by a primarily mitochondrial-dependent mechanism; this increases oxidative damage to lipids and proteins and enhances antioxidant enzyme (CAT and SOD) activities. Increased ROS/RNS production also triggers p38 and ERK1/2 phosphorylation/activation by a redox-sensitive mechanism, as this effect is inhibited by the antioxidant Trolox®, which also prevents other effects caused by retinol-induced ROS/RNS, such as oxidative damage to biomolecules. Activation of p38 resulted in activation of the transcription factor NF-kB, which is inhibited by the p38 pharmacological inhibitors PD169316 and SB203580 and also by p38 knockdown through p38-α and p38-β siRNA transfection. Activated NF-kB (p65) migrates to the cell nucleus and negatively modulates RAGE expression. The NF-kB inhibitor peptide SN50 and p65 knockdown by p65 siRNA transfection blocked RAGE downregulation by retinol, evidencing the role of NF-kB in this effect.

consisting of a heterodimer of p65/p50 retained in the cytoplasm as an inactive tertiary complex associated with inhibitory protein IkBs. After specific stimulation, such as tumor necrosis factor alpha (TNF-α) receptor triggering, IkB phosphorylation by IKKs leads to proteasome degradation of IkB, releasing NF-kB to the nucleus. Once in the nucleus, NF-kB modulates the transcription of innumerous genes, including RAGE. Here, we observed that retinol induced NF-kB activation by a p38-dependent mechanism and this led to decrease of RAGE levels. The use of pharmacological inhibitors of p38 as well as the antioxidant Trolox® reversed the effect of retinol on NF-kB. Also, NF-kB inhibition (by siRNA transfection and pharmacological inhibition) and Trolox® reversed the effect of retinol on RAGE, evidencing the involvement of both p38 and NF-kB in a redox-sensitive signaling pathway triggered by retinol.

Concluding, the results presented here demonstrate for the first time that retinol causes an increase in ROS/RNS production in human lung cancer A549 cells, which leads to NF-kB activation and decreased RAGE expression. We also showed that NF-kB activation is dependent on activation of p38 MAPK, which was induced by retinol in a redox-dependent fashion. RAGE expression has recently been considered a key event in lung cancer development and progression; retinol, on the other hand, was previously considered an antioxidant, anti-cancer agent, but it has been observed to induce deleterious and pro-neoplastic effects. These data reinforce the importance of keeping retinol status within the normal physiologic range and the importance of carefully observing the outcome of vitamin supplementations in epidemiologic and experimental studies. Our findings may also be useful to better understand the molecular mechanisms of RAGE regulation in lungs and retinol action in biological systems.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2013.01.013.

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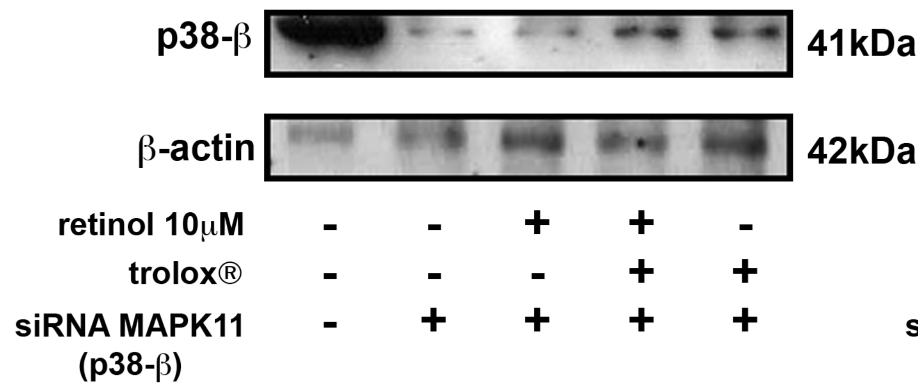
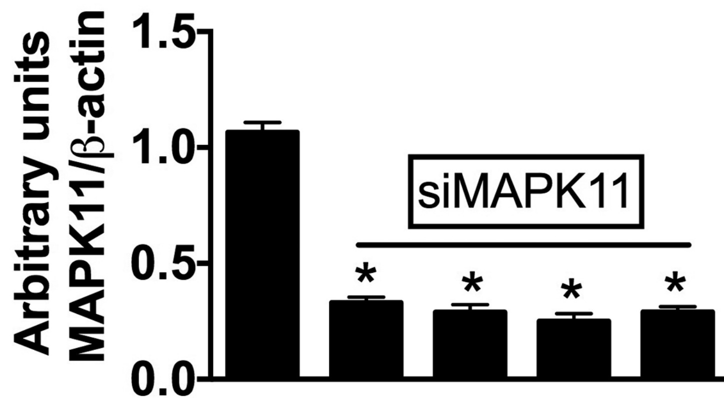
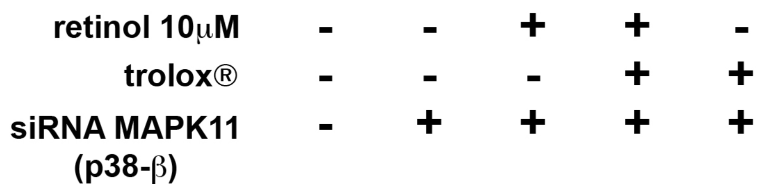
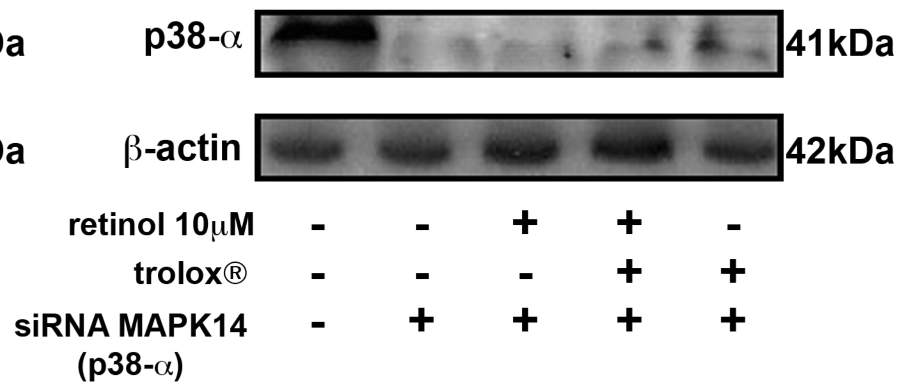
A - Western blot

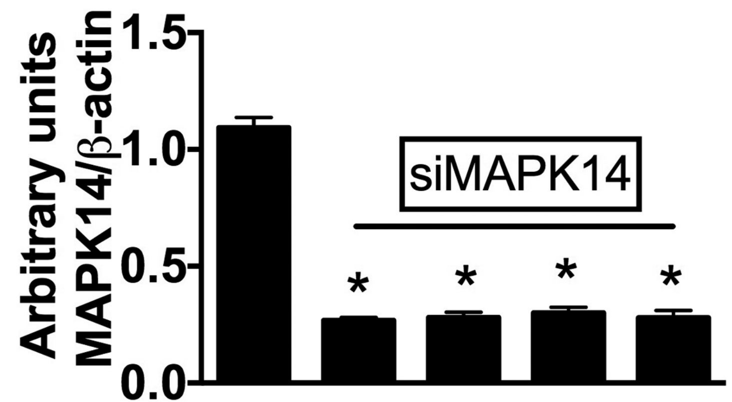


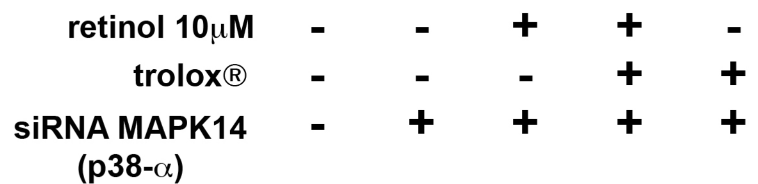




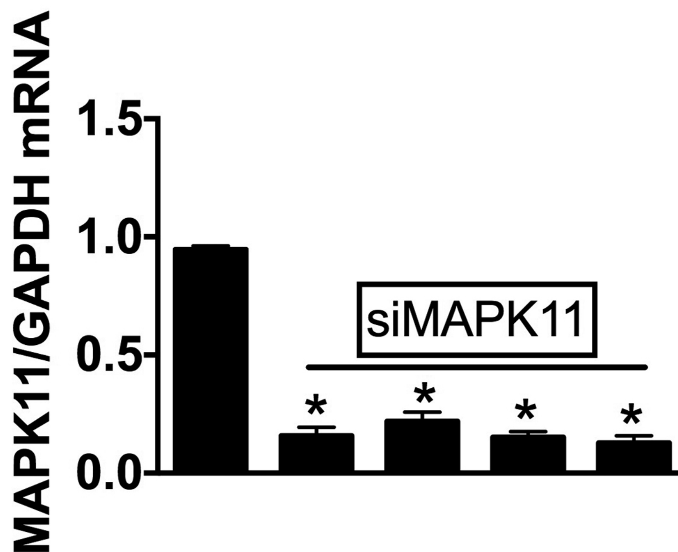
C - Western blot

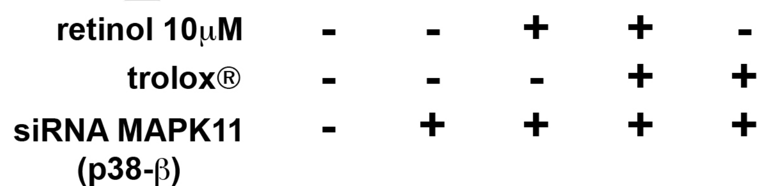




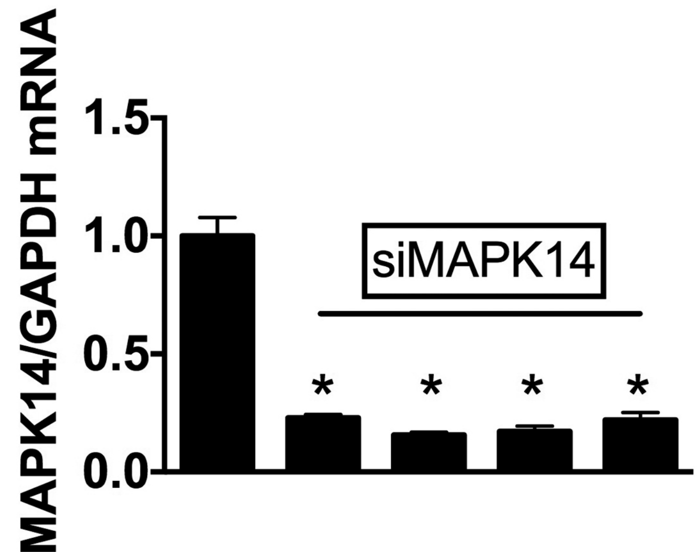


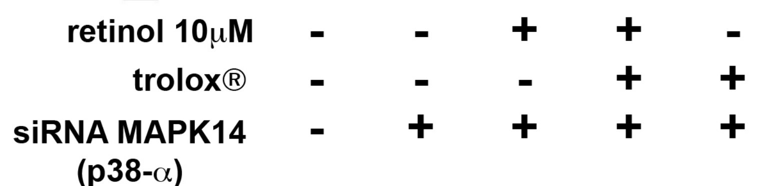
B - qRT-PCR



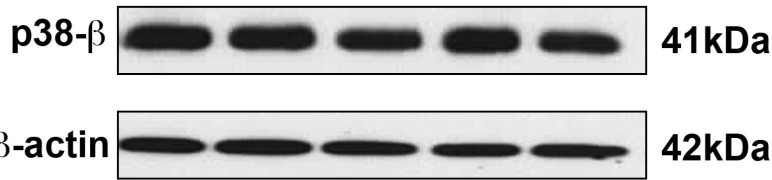


D - qRT-PCR

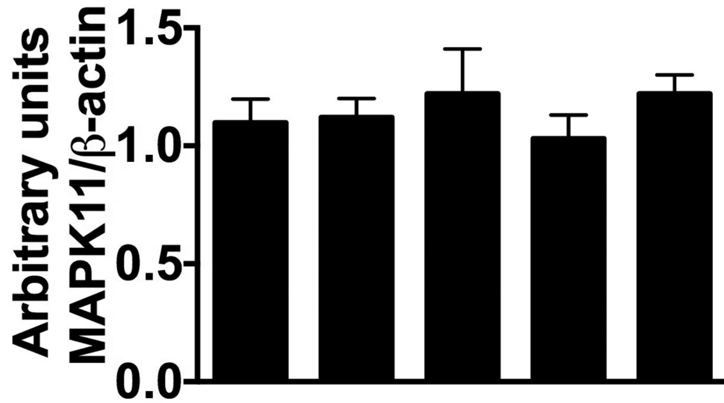




A - Western blot

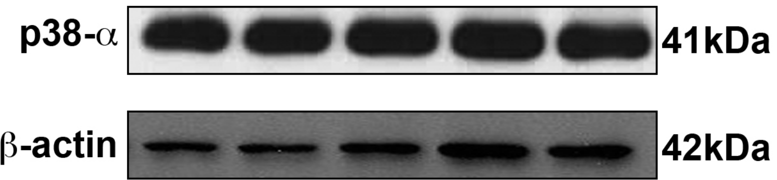


retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+

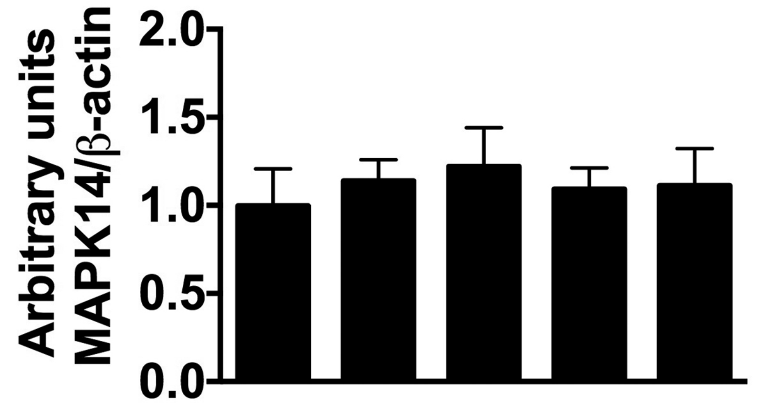


retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+

C - Western blot

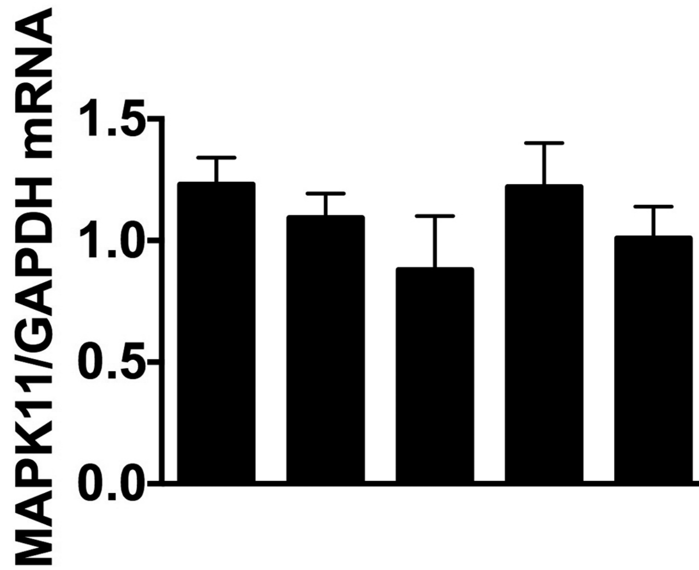


retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+



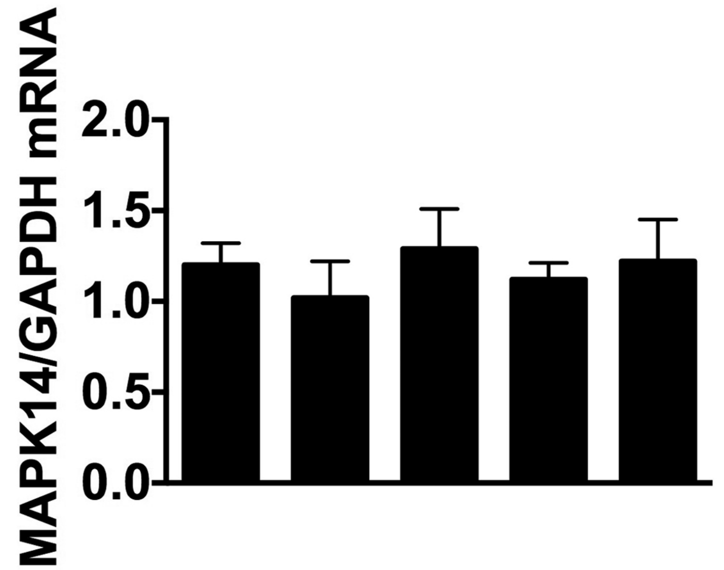
retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+

B - qRT-PCR



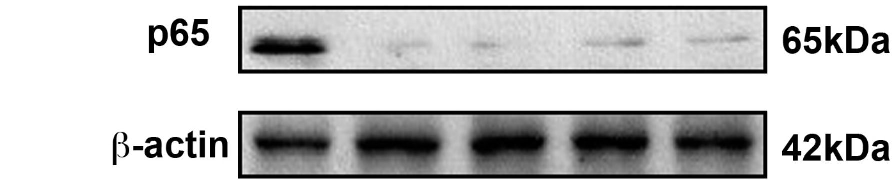
retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+

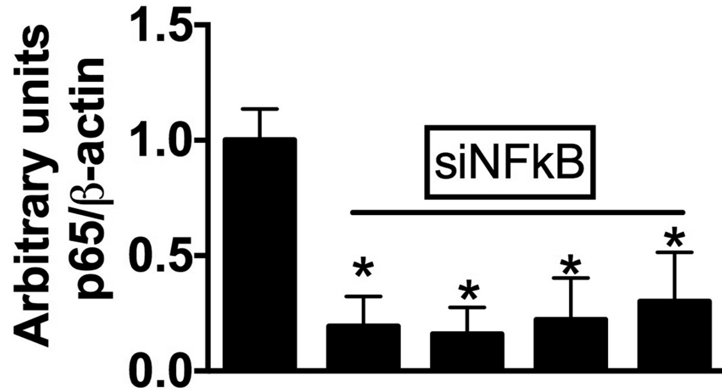
D - qRT-PCR



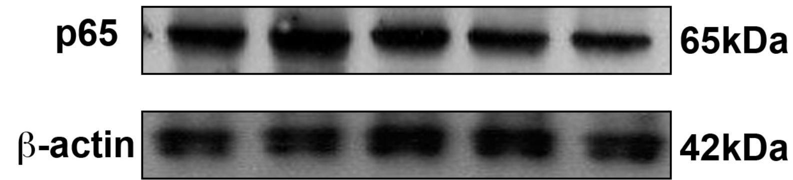
retinol 10μM	-	-	+	+	-
trolox®	-	-	-	+	+
siRNA scrambled	-	+	+	+	+

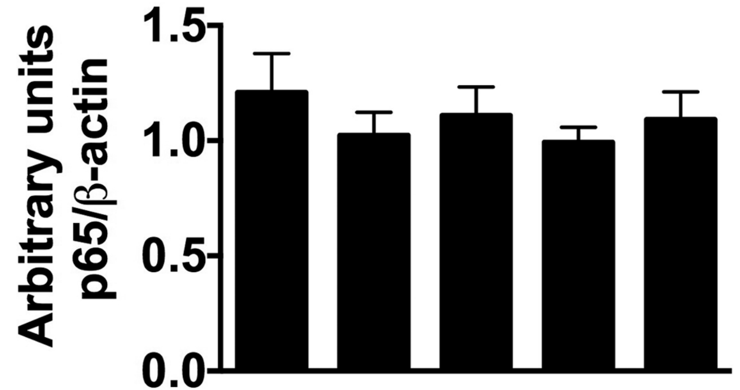
A - Western blot



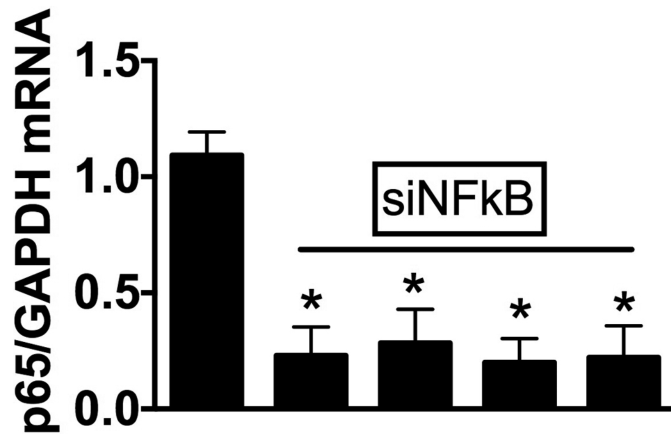


C - Western blot

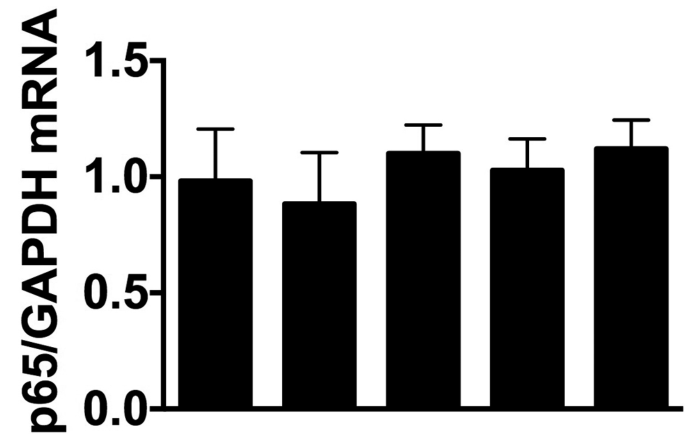




B - qRT-PCR



D - qRT-PCR



A - Table

Primers used in qRT-PCR experiments

Gene	Primers
GAPDH	forward:5' CCATGTTCGTCATGGGTGTGAACCA 3' reverse:5' GCCAGTAGAGGCAGGGATGATGTTG 3'
RAGE	forward:5' ACTGGTGCTGAAGTGTAAGG 3' reverse:5' CCATTCCTGTTCATTGCCTG 3'
MAPK11	forward:5' CAGAAGGTGGCGGTGAAG 3' reverse:5' TCACCAAGTACACTTCGCTG 3'
MAPK14	forward:5' TCTGGATTTTGGACTGGCTC 3' reverse:5' GTTGTAATGCATCCAGTTCAGC 3'
NF-kBp65	forward:5' GACTGCCGGGATGGCTTCTATGAGG 3' reverse:5' CCGCCGCAGCTGCATGGAGAC 3'

PARTE III

4. DISCUSSÃO

As propriedades redox da vitamina A e seus compostos relacionados têm sido objeto de intenso debate recentemente. Uma das características mais importantes da vitamina A é a sua capacidade de ser uma molécula antioxidante ou pró-oxidante assim como outras moléculas como a vitamina C e licopeno. A propriedade pela qual então a vitamina A irá agir dependerá de situações como: concentração no meio celular, tipo celular, momento metabólico entre outras; e desta forma ela poderá exercer seus efeitos antioxidantes ou pró-oxidantes. Inicialmente, devido à sua grande importância dentro dos sistemas biológicos, os retinóides eram utilizados como moléculas antioxidantes (Bollag 1983). Essa crença estimulou o uso de retinóides como antioxidantes em suplementos nutricionais para a prevenção ou até mesmo o tratamento de diversas doenças (O'Byrne e Blaner 2013).

Sabe-se que os radicais livres e outras formas de espécies reativas estão diretamente correlacionados com maior risco e incidência de doenças de ordem respiratória, neurológica e cardiovascular (MacNee 2001; MacNee e Rahman 2001). Em alguns casos, a deficiência de retinol no soro foi avaliada nestas doenças e a suplementação com retinóides ou administração farmacológica foi propostas como terapias antioxidantes para o tratamento e / ou prevenção de tais condições (Sommer e Vyas 2012). Para os pulmões, por exemplo, a deficiência de vitamina A pode induzir enfisema pulmonar, através da diminuição do conteúdo de elastina e da síntese de surfactante (Dirami, Massaro et al. 2004). No entanto, neste contexto, estudos já demonstraram que a suplementação alimentar de vitamina A aumentou 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) e por

esses motivos os estudos tiveram que ser interrompidos devido ao aumento da mortalidade relatada (Omenn, Goodman et al. 1996). Os autores sugeriram que os efeitos observados nesses estudos poderiam estar relacionados as propriedades pró-oxidantes da vitamina A, as quais estariam sendo impostas no meio pela suplementação com vitamina A.

Recentemente, diversos trabalhos têm demonstrado que a vitamina A pode exercer um significativo potencial pró-oxidante em diferentes sistemas, como "*in vitro*", em cultura de células e em modelos animais (Klamt, Dal-Pizzol et al. 2003; Gelain, Casali et al. 2005; Klamt, Dal-Pizzol et al. 2008; de Oliveira, da Rocha et al. 2011). Ambos beta-caroteno e seus metabólitos: retinol, retinal e ácido retinóico , são capazes de aumentar a formação do radical $OH^{\bullet}$ em sistemas *"in vitro"* contendo ferro e H_2O_2, através da reação de Fenton. Em cultura celular a elevação da concentração de retinol também estaria associada a um significativo potencial pró-oxidante, induzindo alterações redox em biomoléculas. Já em modelos animais, a suplementação com vitamina A é capaz de gerar alterações comportamentais, tais como, induzir ansiedade e diminuir atividades locomotoras e exploratórias de ratos Wistar suplementados com doses consideradas pela literatura como terapêuticas (de Oliveira, da Rocha et al. 2011). No tecido pulmonar, nós recentemente demonstramos, que a suplementação com vitamina A induz alterações em parâmetros redox tais como: aumento nos níveis de carbonilação de proteínas e de peroxidação lipídica, e diminuição na concentração de grupamentos tióis reduzidos (Pasquali, Gelain et al. 2009a; Pasquali, Gelain et al. 2009b; Pasquali, Schnorr et al. 2010). Além disso, nós também observamos que a suplementação de vitamina A em ratos Wistar fêmeas grávidas, é capaz de induzir estresse oxidativo nos pulmões de seus

filhotes, demonstrando assim possíveis riscos que uma suplementação com vitamina A pode acarretar para a prole (Pasquali, Schnorr et al. 2010).

No presente trabalho, nós investigamos o efeito do tratamento com retinol, o qual é considerado o principal metabólito da vitamina A, na linhagem A549 de carcinoma de pulmão de não-pequenas células. Nós avaliamos se o tratamento com retinol nestas células alterava o ambiente redox, assim como se induzia a ativação de vias de sinalização envolvidas na modulação do receptor para produtos finais avançados de glicação (RAGE). A linhagem A549 é muito utilizada pela literatura, tanto para experimentos envolvendo a biologia do câncer, como para avaliar os mecanismos de funcionamento das células pulmonares (Forbes e Ehrhardt 2005). Nós observamos que o tratamento com retinol em diferentes doses, as quais podem ser atingidas fisiologicamente, nesta linhagem de células induziu um estado pró-oxidativo nas mesmas. Encontramos aumento nos níveis de peroxidação lipídica, carbonilação de proteínas, assim como alterações nas atividades das enzimas antioxidantes catalase (CAT) e superóxido dismutase (SOD), os quais são utilizados como marcadores característicos de estresse oxidativo.

Apesar desses efeitos característicos de estresse oxidativo terem sido observados com o tratamento com retinol, as células A549 não tiveram a sua viabilidade alterada. Pelo contrário, nas doses de 10 μM e 20 μM de retinol, houve um aumento no número de células viáveis. Como em outros trabalhos nós já havíamos observado que a suplementação com vitamina A induzia um estado de estresse oxidativo, nós decidimos aqui tentar elucidar por qual mecanismo neste modelo o tratamento com retinol estava induzindo um estado pró-oxidativo nestas células. Para isso nós avaliamos a produção de espécies reativas do oxigênio pelo tratamento com retinol em tempo real, assim como a participação das mitocôndrias e

da enzima NADPH-oxidase na produção de espécies reativas do oxigênio. Os resultados encontrados foram que o tratamento com retinol induziu um aumento na produção de espécies reativas do oxigênio, sendo que as mitocôndrias tiveram uma participação importante neste aumento na produção das espécies reativas através da produção $O_2^{-\bullet}$. Já a produção de $O_2^{-\bullet}$ pela enzima NADPH-oxidase não foi alterada pelo tratamento com retinol.

Desta maneira ficou evidenciado que o tratamento com retinol foi capaz de induzir uma alteração na atividade mitocondrial. A atividade mitocondrial eficaz está associada ao correto funcionamento da cadeia transportadora de elétrons por meio de seus complexos (Kroemer, Galluzzi et al. 2007). Alterações nas atividades dos complexos da cadeia transportadora de elétrons pode favorecer a redução parcial do O_2 nas mitocôndrias, levando então a um aumento na produção de $O_2^{-\bullet}$, já que a taxa de transferência para o O_2 (o qual é o aceptor final de elétrons na cadeia transportadora mitocondrial) no complexo IV pode estar alterada (Kroemer, Galluzzi et al. 2007). Este aumento na produção de $O_2^{-\bullet}$ pelo tratamento com retinol pode estar relacionado com o aumento na atividade de SOD que nós observamos, isto porque ele é o seu principal ativador alostérico (Shimizu, Kobayashi et al. 1984). Essa modulação na atividade de SOD, pode levar a um desequilíbrio na relação SOD/CAT. Um aumento da atividade de SOD ocorrendo simultaneamente com diminuição da atividade de CAT pode resultar em um aumento na concentração de H_2O_2, isto devido à sua produção a partir de $O_2^{-\bullet}$ através da atividade de SOD estar aumentada e eliminação de H_2O_2 pela CAT estar diminuída (Shimizu, Kobayashi et al. 1984).

Essa alteração que um desbalanço na relação SOD/CAT causa pode aumentar ainda mais a produção de espécies reativas do oxigênio presentes no ambiente celular,

as quais podem atacar proteínas, danificando as mesmas, levando à formação de proteínas carboniladas, que foi o efeito verificado com o tratamento com retinol nas células A549. O aumento dos níveis de carbonilação de proteínas reflete os níveis de oxidação protéica nas células. A carbonilação de proteínas pode ser gerada através da quebra de estrutura da proteína, retirada de um átomo de hidrogênio no carbono alfa, ataque a cadeia lateral de diversos aminoácidos (como Lisina, Arginina, Prolina), pela formação dos adutos de Michael entre os resíduos de lisina, histidina e cisteína, e através da ação de produtos reativos da peroxidação lipídica. A glicação e glicoxidação nos grupos amino de Lisina pode levar a formação dos chamados produtos finais avançados de glicação (AGEs), os quais também podem levar a carbonilação de proteína. A geração então de radicais protéicos pode levar a formação de outros radicais, os quais podem causar danos a outras biomoléculas, gerando então uma cadeia de reações de oxidação/redução (Bindoli, Fukuto et al. 2008). Muitos danos a proteínas são irreparáveis e podem levar a consequências como dimerização ou agregação, desdobramento ou mudanças conformacionais expondo resíduos mais hidrofóbicos ao ambiente aquoso celular, perda de estrutura ou atividade funcional, alteração no *turnover*, modulação na regulação gênica tanto a nível de expressão quanto de regulação, modulação da sinalização celular podendo levar a indução de apoptose ou proliferação. Aqui nós observamos que o tratamento com retinol ocasionou um aumento na carbonilação de proteínas, sendo que todos os efeitos pró-oxidativos mediados pelo tratamento com retinol nas células A549 foram bloqueados através do co-tratamento com Trolox® (análogo hidrofílico da vitamina E), o qual é uma molécula antioxidante.

Além do tratamento com retinol induzir um estado pró-oxidativo nas células A549 afetando de modo geral a homeostase celular, ele também induziu a diminuição

da expressão e do imunoconteúdo do receptor para produtos finais avançados de glicação (RAGE). Este receptor é altamente expresso nas células pulmonares, sendo que diversos trabalhos demonstram que a sua integridade e expressão é fundamental para a manutenção da homeostase do tecido pulmonar (Queisser, Kouri et al. 2008). O decréscimo do conteúdo de RAGE está correlacionado com a progressão de diversas doenças pulmonares como: doença pulmonar obstrutiva crônica, fibrose pulmonar e câncer de pulmão (Hsieh, Schafer et al. 2003; Queisser, Kouri et al. 2008; Wang, Cui et al. 2012). Aqui nós observamos que a diminuição de RAGE, pelo tratamento com retinol, foi mediada pelo aumento da produção de espécies reativas, sendo que o co-tratamento com Trolox® foi capaz de bloquear o efeito do retinol, demonstrando assim que o conteúdo de RAGE nas células estava sendo modulado de maneira redox dependente.

A importância do RAGE para a homeostase do tecido pulmonar é devido ao tipo de interação a seus ligantes e resposta celular que esse receptor pode ter no tecido pulmonar. Recentemente vêm sendo sugerido a utilização da expressão do RAGE para diagnóstico de doenças respiratórias, assim como o seu uso no tratamento das mesmas e isso se deve ao fato de ele ter sua expressão diminuída nas doenças respiratórias (Wang, Cui et al. 2012). Diversos agentes endógenos com potencial mitogênico, como os produtos finais de glicação avançada (AGEs), proteínas da família S100B, e HMGB1 podem interagir com o RAGE. Sua ligação ao RAGE pode estimular a proliferação celular, devido ao fato da ativação do RAGE ser capaz de induzir alterações intracelulares relacionadas ao ciclo celular em diversos tipos celulares (Huttunen, Kuja-Panula et al. 2002; Cortizo, Lettieri et al. 2003). Além disso, a ativação de RAGE por essas moléculas também pode induzir processos inflamatórios, invasividade e metástase de tumores (Degryse, Bonaldi et al. 2001).

Por este motivo a homeostasia desse receptor no pulmão é fundamental para a manutenção da fisiologia deste tecido. Como o tratamento com retinol diminuiu o conteúdo e expressão desse receptor, o qual é de suma importância para o pulmão, nós então decidimos avaliar por qual mecanismo o tratamento com retinol estaria causando este efeito.

A regulação do RAGE é descrita na literatura como uma regulação tecido dependente(Su, Looney et al. 2009; Buckley e Ehrhardt 2010). Com exceção do tecido pulmonar, a expressão do RAGE é regulada positivamente em um grande número de tipos de células em condições patológicas. Estas observações representam, provavelmente, uma das principais razões por que a expressão do RAGE e a sinalização mediada por ele, em sua maioria, têm sido investigadas no contexto da fisiopatologia de estados inflamatórios crônicos e câncer (Wendt, Bucciarelli et al. 2002). Diversos trabalhos já demonstraram a participação das proteínas cinases ativadoras mitogênicas (MAPK) - p38, ERK 1/2 e JNK na modulação do RAGE em diferentes tipos de células e condições patológicas (Bucciarelli, Wendt et al. 2002a; Bucciarelli, Wendt et al. 2002b; Bucciarelli, Sramek et al. 2002). Porém no tecido pulmonar, a participação dessas vias na modulação do RAGE ainda não havia sido investigada, principalmente devido ao fato de esse receptor ser expresso de maneira constitutiva nesse tecido.

Como a regulação de RAGE, em estados inflamatórios e câncer, se dá pela ativação das MAPKs, e que as espécies reativas do oxigênio, em muitos desses casos, podem participar da ativação dessas cinases, nós então avaliamos a participação das MAPKs na diminuição da expressão e imunoconteúdo de RAGE ocasionada pelo tratamento com retinol nas células A549. Nós observamos que o tratamento com retinol, em períodos curtos, levou a ativação de ERK 1/2 e p38. Essa ativação foi

mediada pela fosforilação dessas proteínas. A fosforilação de ERK 1/2 aumentou ao longo do tempo analisado, o qual foi de 60 minutos. Já a fosforilação de p38, apresentou um pico após o tratamento com retinol, o qual foi observado no tempo de 30 minutos. Diversos trabalhos já demonstraram que essas cinases podem ser ativadas de maneira redox dependente, sendo que a ativação tanto de ERK 1/2 como de p38 pode também estar associada a ativação do ciclo celular, devido ao fato delas estarem envolvidas no controle de cascatas de sinalização que levam a fosforilação de ciclinas e outras cinases que participam da regulação do ciclo celular.

A ativação dessa cinases pelo tratamento com retinol se mostrou ser redox dependente em nosso modelo, pois quando as células foram co-tratadas com Trolox®, a ativação dessas cinases, via sua fosforilação, foi bloqueada. Surpreendentemente, o co-tratamento com Trolox®, além de inibir a ativação da ERK 1/2 e da p38, também bloqueou a diminuição da expressão e imunoconteúdo do RAGE. Devido o fato dessas cinases estarem envolvidas na modulação do RAGE em outros tecidos, nós avaliamos então a participação delas na modulação do RAGE mediada pelo tratamento com retinol. Através da utilização de inibidores específicos para ERK 1/2 e p38, nós evidenciamos que a ativação através da fosforilação de p38 mediada pelo tratamento com retinol estava diretamente influenciando a regulação da expressão e imunoconteúdo do RAGE.

Com o intuito de esclarecer o mecanismo pelo qual o retinol estava alterando o conteúdo do RAGE em nosso modelo, decidimos silenciar transitoriamente as células para a proteína p38. Nós silenciamos as subunidades p38-α (MAPK14) e p38-β (MAPK11) com siRNA específico para ambas. O silenciamento das subunidades de p38 bloqueou o efeito do tratamento do retinol sobre a expressão e imunoconteúdo do RAGE. Esses resultados, junto com os resultados dos inibidores para a p38,

evidenciaram a real participação da p38 na modulação do RAGE mediado pelo tratamento com retinol, o qual induziu a fosforilação de p38 de maneira redox dependente.

A ativação de p38 é amplamente estudada principalmente no campo da inflamação. Diversos estressores celulares, tanto extracelulares como citocinas quanto intracelulares como as espécies reativas do oxigênio, são descritos como ativadores dessa proteína. O mecanismo de ação da p38 pode envolver a interação com outras vias de sinalização intracelular. Ela pode interagir com fatores de transcrição como NF-kB, Nrf2 e AP-1, ativando os mesmos. Dentre esses fatores de transcrição, o fator de transcrição NF-kB é amplamente descrito com um dos principais moduladores do RAGE em estados inflamatórios em diversos tecidos (Paimela, Ryhanen et al. 2012; Salminen, Hyttinen et al. 2012; Salminen, Kauppinen et al. 2012). O NF-kB normalmente está situado no citoplasma ligado a IkBα, a qual é uma proteína chaperona que inibe a ativação do NF-kB. O NF-kB só é ativado quando uma IkB cinase, contendo as subunidades catalíticas IkB cinase 1 e IkB cinase 2, fosforila a IkBα, causando assim a sua ubiquitinação e proteólise, liberando assim o NF-kB para que o mesmo possa a vir translocar-se para o núcleo da célula e iniciar a transcrição gênica.

Como a ativação de NF-kB pode regular a expressão do RAGE, nós também investigamos a participação da ativação desse fator de transcrição na modulação do RAGE mediada pelo tratamento com retinol nas células A549. O tratamento com retinol induziu a translocação do NF-kB para o núcleo das células, esse efeito também demonstrou ser mediado por um mecanismo redox-dependente, pois quando as células foram co-tratadas com Trolox® houve a inibição da translocação do NF-kB para o núcleo das células.

Para elucidar se realmente o NF-kB também estava envolvido na modulação do RAGE mediada pelo tratamento com retinol, nós utilizamos um inibidor farmacológico de NF-kB, o SN50, o qual inibe a sua translocação para o núcleo. O co-tratamento das células com retinol e inibidor acabou bloqueando o efeito do retinol sobre a expressão e imunoconteúdo do RAGE, demonstrando assim que o fator de transcrição NF-kB estava, assim como a p38, envolvido na modulação do RAGE mediada pelo retinol. Além disso, quando nós utilizamos os inibidores de p38 como co-tratamentos com o retinol, os mesmos também acabaram por inibir a translocação do NF-kB para núcleo das células. Desta maneira demonstramos que a modulação do RAGE mediada pelo tratamento com retinol era dependente de uma relação entre a ativação de p38 e NF-kB.

Todavia, para confirmar a participação do NF-kB na modulação do RAGE mediada pelo tratamento com retinol nas células A549, nós silenciamos as células para a subunidade de p65 do fator de transcrição NF-kB e realizamos o tratamento com retinol. A subunidade p65 do fator de transcrição NF-kB é a responsável por translocar para o núcleo das células e agir como fator de trancrição. O silenciamento dessa subunidade foi feito através de siRNA específico para ela. Através do silenciamento, nós verificamos que realmente a ativação do fator de transcrição NF-kB estava envolvida na modulação do RAGE por retinol, pois a diminuição da expressão e imunoconteúdo do RAGE mediada pelo tratamento com retinol foi bloqueada quando as células A549 foram silenciadas para a subunidade p65 do NF-kB.

Portanto, neste trabalho, demonstramos que o retinol é capaz de diminuir a expressão e imunoconteúdo do RAGE em células A549 através de um mecanismo redox-dependente o qual envolve a ativação da MAPK p38 e do fator de transcrição

NF-kB. Esta foi a primeira vez na literatura que este mecanismo foi demonstrado, ou seja, que a ativação de NF-kB induzida pela p38 ocasiona uma diminuição da expressão e imunoconteúdo do RAGE. Este mecanismo nas células pulmonares parece ter um efeito oposto a outros tecidos no corpo, pois a ativação de NF-kB mediada pelo tratamento com retinol nas células A549, reduziu o conteúdo do RAGE, enquanto que em outros tecidos, quando ativado, o NF-kB está relacionado com o aumento do RAGE (Brune, Muller et al. 2013; Meneghini, Bortolotto et al. 2013).

Concluindo, os dados por nós encontrados aqui neste trabalho, chamam a atenção e reforçam ainda mais a literatura no contexto de se manter uma atenção especial à suplementação com vitamina A e seus efeitos no organismo. O excesso de vitamina A pode apresentar efeitos deletérios para o organismo ou para os tecidos, alterando o seu estado redox, assim como a homeostasia dos tecidos. O completo e complexo efeito da suplementação com vitamina A ainda permanece por ser elucidado, e desta forma as peculiaridades do mecanismo de ação da vitamina A em sistemas biológicos necessitam de mais estudos para que sejam melhor compreendidas.

5. CONCLUSÕES

Baseando-se nos resultados obtidos no presente trabalho, podemos concluir que:

- O tratamento com retinol, em concentrações que podem ser encontradas fisiologicamente, induziu um aumento na peroxidação lipídica e carbonilação de proteínas. Além disso ele alterou o estado redox de grupamentos sulfridril protéicos e não-protéicos. As atividades das enzimas antioxidantes: superóxido dismutase (SOD) e catalase (CAT) também se alteraram com o tratamento com retinol, aumentando e diminuindo respectivamente. Desta maneira, o tratamento com retinol se mostrou ser pró-oxidativo em nosso modelo;
- O tratamento com retinol aumento a produção de espécies reativas, sendo que ele induziu aumento na produção mitocondrial do radical $O_2^{-\bullet}$, sem alterar a produção do mesmo radical pela enzima NADPH-oxidase. Portanto esse aumento na produção de radical $O_2^{-\bullet}$ pode ter favorecido o estresse oxidativo observado através dos danos oxidativos a biomoléculas;
- Mesmo alterando o ambiente redox das células para um estado pró-oxidativo o tratamento com retinol não diminuiu a viabilidade celular, pelo contrário, nas doses de 10 μM e 20 μM ele aumentou a viabilidade das células;
- O tratamento com retinol reduziu a expressão e o imunoconteúdo do receptor para produtos finais avançados de glicação (RAGE), de maneira dose dependente. Essa diminuição do RAGE mostrou-se ser redox dependente, pois o co-tratamento com Trolox ® bloqueou o efeito mediado pelo retinol;

- As proteínas cinases ativadoras mitogênicas (MAPK) - p38 e ERK 1/2 foram ativadas, pelo tratamento com retinol, através da sua fosforilação. Esse efeito do retinol foi redox-dependente também, pois o co-tratamento com Trolox® bloqueou a ativação dessas proteínas. Além disso, a ativação de p38 mostrou-se envolvida na modulação do RAGE, quando a mesma foi silenciada o efeito do retinol sobre o RAGE foi bloqueado;
- O tratamento com retinol, de maneira redox dependente, também levou à ativação do fator de transcrição NF-kB, o qual acabou por regular a expressão e imunoconteúdo do RAGE. A ativação de NF-kB foi dependente da ativação da p38, demonstrando assim que a interação entre essas duas vias foi responsável pela diminuição da expressão e imunoconteúdo do RAGE mediada pelo tratamento com retinol;
- Devido ao importante papel do RAGE para homeostase do tecido pulmonar, e com os dados obtidos neste trabalho, é de suma importância trabalhos que venham a elucidar ainda mais os mecanismos de ação e efeitos da vitamina A sobre os sistemas biológicos.

6. PERSPECTIVAS

A partir dos resultados obtidos com esta tese, pretendemos continuar investigando as consequências moleculares da suplementação com vitamina A em modelo de cultivo celular. Pretendemos ampliar os estudos a outras linhagens de células pulmonares assim como em culturas primárias do tecido pulmonar, com o intuito de compreender melhor o mecanismo pelo qual o tratamento com retinol induz aumento na produção de espécies reativas do oxigênio e modula o RAGE. Análise da translocação do RAGE para mitocôndrias; da atividade dos complexos da cadeia transportadora de elétrons assim como seu imunoconteúdo e expressão deverão ser estudados, afim de esclarecer melhor o aumento da produção de $O_2^{-\bullet}$ pelas mitocôndrias.

O efeito da ativação de ERK 1/2 mediado pelo tratamento com retinol, também deverá ser estudado, afim de esclarecer a participação desta via no aumento da viabilidade celular observada neste trabalho. Portanto, esses estudos serão necessários para podermos ter uma resposta mais completa dos possíveis efeitos que uma suplementação com vitamina A pode causar, principalmente no que diz respeito ao tecido pulmonar.

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ANEXOS

ANEXO I

"Vitamin A supplementation to pregnant and breastfeeding female rats induces oxidative stress in the neonatal lung"

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Vitamin A supplementation to pregnant and breastfeeding female rats induces oxidative stress in the neonatal lung

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ABSTRACT

Vitamin A is an essential micronutrient that regulates many biological processes through modulation of retinoic acid receptor-responsive genes. Vitamin A acts as a systemic antioxidant, participating in the modulation of diverse redox mechanisms involved in physiological and pathological processes. Different studies, however, observed that vitamin A and other retinoids may induce pro-oxidant/deleterious actions under certain conditions, leading to impairment of brain and lung function. Here, we studied the effect of vitamin A treatment at oral doses of 100 IU/kg, 200 IU/kg, and 300 IU/kg to female rats (*Rattus norvegicus*) during pregnancy and lactation on oxidative parameters of lungs from the offspring vitamin A supplementation induced increases in lipoperoxidation, protein carbonyl, activities of the antioxidant enzymes superoxide dismutase and catalase (200 IU/kg, and 300 IU/kg), and decreased sulphydryl protein (500 IU/kg) content in the neonatal lung.

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1. Introduction

Vitamin A (retinol, retinoic acid) plays a key role in many essential biological processes. It is essential for embryonic development and the regulation of proliferation and differentiation of many cell types [1]. The discovery of nuclear receptors for retinoic acid and other retinoids has provided a conceptual basis to explain how these compounds preside over a large network of gene activation processes [2]. The physiological actions of vitamin A are generally described as being mediated by nuclear receptors from steroid/thyroid superfamily.

Studies have described a protective role of vitamin A in several diseases, which was related to its ability to scavenge toxic forms of oxygen and other free radicals mainly in rats and mice [3]. Some patients with acute myeloid leukemia respond to very large doses of all-*trans*-retinyl palmitate of the order 50,000 IU/kg/day. The role of vitamin A is also particularly important for embryogenesis [4]. Hypovitaminosis A may lead to failure of embryo segmentation and growth, ceasing vascularization and impairing development in rats and guinea pigs (*Cavia porcellus*) [5]. Hypervitaminosis A, on the other hand, is teratogenic in many model systems [6]. Human susceptibility to the teratogenic effects of vitamin A have been associated to even a single supplement of 500,000 IU in the first 2 months or at 25,000 IU daily throughout pregnancy. In addition, excess of vitamin A can disrupt membranes and is hepatotoxic in adults [7].

Our previous results demonstrated that vitamin A supplementation (retinol at 7 μM) induces oxidative damage in biomolecules [8–10], upregulation of antioxidant enzymes [11,12], preneoplasic transformation [13], and activation of phosphorylation signaling pathways in cultured Sertoli cells [14–16]. Thus, vitamin A may promote oxidative stress and modulate redox-dependent processes in tissues. Oxidative stress may follow from the generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS) through increased production or decreased antioxidant capacity, or when such factors are combined [17]. Oxidative stress may contribute to the pathogenesis of diffuse lung diseases (DLD) [18].

Data regarding effects of vitamin A supplementation in human lung development are limited. Given that vitamin A supplementation causes oxidative stress in rat lungs at doses from 1000 IU/kg/day to 9000 IU/kg/day, as we previously demonstrate [19,20], the aim of this study was to investigate the effects of vitamin A supplementation at lower dosages of 100 IU/kg, 200 IU/kg, and 500 IU/kg on parameters of oxidative stress in neonatal rat lung from dams treated with vitamin A supplementation during pregnancy and lactation.

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2. Materials and methods

2.1. Animals

Nulliparous female Wistar rats (*Rattus novergicus*) at 90 days of age and 200–250 g in weight were obtained from an in-house breeding colony. All animals used in this study were healthy and free of pathogens. Dams were caged in groups of five animals with free access to standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and water. They were maintained on a 12-h light–dark cycle (7:00–19:00 h) at 23 ± 1 °C. The females were selected at their sexual receptive phase of the estrous cycle by vaginal smear and were placed individually with a single mature male of proven fertility from the same colony, overnight. Successful mating was regarded as the presence of vaginal plug and/or viable sperm at vaginal smear, and this was designed as day 0 of gestation. All housing, handling and euthanasia procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Treatment

Pregnant dams were randomly selected for daily treatment throughout gestation and lactation (21 days of gestation and 21 days of lactation). Vitamin A was given at night in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The number of females in each group varied between 5 and 12 animals and the number of pups per litter ranged from 7 to 12. All newborns were analyzed regardless of gender.

Dams were treated with vehicle (0.15 M saline; control group), 100 IU/kg, 200 IU/kg and 500 IU/kg of retinol palmitate (Arovit®; a water-soluble form of vitamin) orally, via a metallic gastric tube (gavage) in a maximum volume of 0.6 ml. Adequate measures were taken to minimize pain or discomfort. The recommended daily intake for humans is 5000 IU/day (1.5 mg/day of retinol) of vitamin A. The doses used in this study are doses of supplementation that can be easily achieved in humans through the ingestion of foods fortified with vitamin A. Furthermore, these dosages are considered safe for humans [6] and this intake of foods fortified with vitamins is observed mainly in developed countries or developing countries.

2.3. Drugs and reagents

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche, São Paulo, SP, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A administration solutions were prepared daily, protected from light exposure and temperature.

2.4. Lung extraction and samples preparation

The animals were euthanized by decapitation 24 h after the last treatment. Lungs were quickly dissected out on ice and stored at −80 °C for subsequent biochemical analyses. Samples were homogenized in 50 mM phosphate buffer (pH 7.0) and the lysate was clarified by centrifugation (700 × *g*, 5 min) to generate the crude supernatant fraction used in all biochemical assays described. Results were normalized by the protein content using bovine albumin as standard [21].

2.5. Thiobarbituric acid reactive species (TBARS) assay

As an index of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state [22]. Briefly, the samples were mixed with 0.6 ml of 10% trichloroacetic acid (TCA) and 0.5 ml of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as nmol TBARS/mg protein.

2.6. Measurement of protein carbonyls

Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described [23]. Briefly, proteins were precipitated by the addition of 20% TCA, resolubilized in DNPH, and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.7. Measurement of protein thiol content

Protein thiol content was analyzed to estimate oxidative alterations in proteins. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,50-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol were added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min [24]. Results are expressed as mmol SH/mg protein.

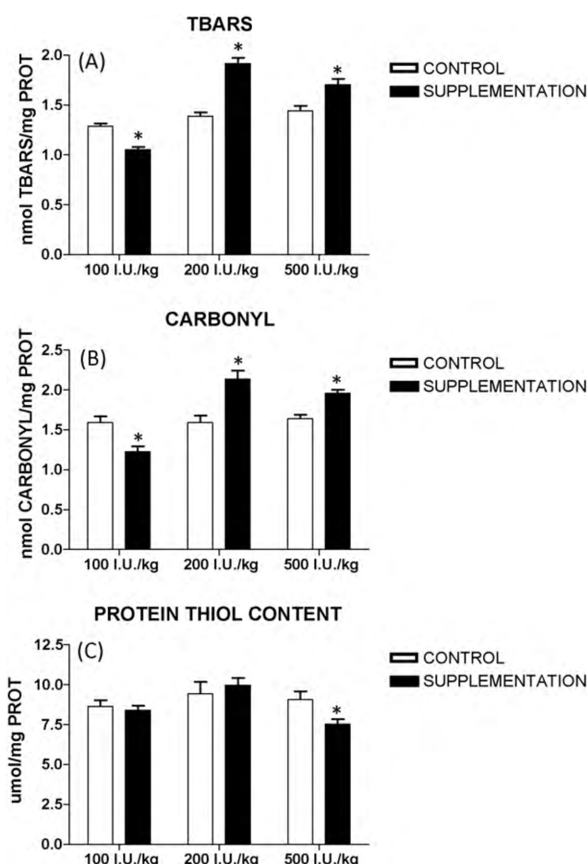

Fig. 1. Effects of vitamin A supplementation on lipid peroxidation (A), protein carbonylation (B), protein thiol content (C) in the offspring lungs. Data are mean ± SEM (n = 5–12 L). *p < 0.05 (one-way ANOVA followed by the post hoc Tukey's test).

2.8. Antioxidant enzyme activities estimations

Catalase (EC 1.11.1.6; CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [25], and the results are expressed as units of CAT/mg protein. Superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [26], and the results are expressed as U SOD/mg protein. A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A supplementation upon these two free radical-detoxifying enzymes that work in sequence converting superoxide anion to water. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

2.9. Statistical analysis

Biochemical results are expressed as means ± standard error of the mean (SEM); p values were considered significant when p < 0.05. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary (GraphPad Prism 4 – Software). We used the average of each litter for statistical analysis.

3. Results

We first analyzed the effect of vitamin A supplementation on lipoperoxidation in the neonatal lung as determined by TBARS levels. Vitamin A supplementation at 100 IU/kg decreased TBARS levels (Fig. 1A); however, at 200 IU/kg and 500 IU/kg, vitamin A supplementation induced an increase in the levels of TBARS, indicating an increase in lung lipoperoxidation (Fig. 1A). Treatment induced a similar effect on lung protein carbonylation. At 100 IU/kg, vitamin A administration induced a decrease in the levels of protein carbonyls, whereas at 200 IU/kg and 500 IU/kg carbonylation levels

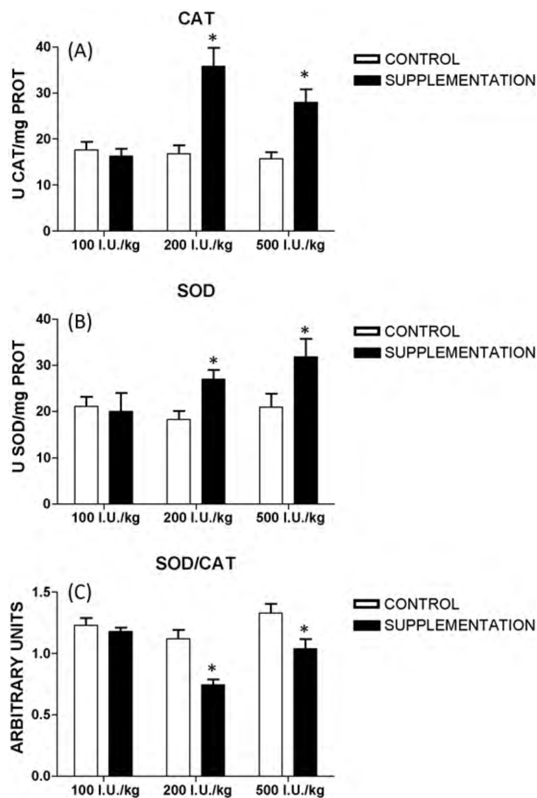

Fig. 2. Effects of vitamin A supplementation on SOD (A), CAT (B), activity in the offspring lungs. (C) The lung SOD/CAT ratio. Data are mean ± SEM (n = 5–12 L). *$p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

were found to be increased (Fig. 1B). Finally, protein thiol content was observed to be decreased by vitamin A supplementation only at 500 IU/kg (Fig. 1C).

We next analyzed the enzymatic activity of CAT, which is involved on hydrogen peroxide (H_2O_2) detoxification, and SOD, which metabolizes superoxide radicals. CAT activity was enhanced in newborns that receiving vitamin A supplementation at doses of 200 IU/kg and 500 IU/kg (Fig. 2A). These same doses induced a stimulation on SOD activity as well (Fig. 2B). Plotting a ratio between the activities of SOD and CAT showed that, besides increasing the activities of SOD and CAT, vitamin A supplementation at 200 IU/kg and 500 IU/kg caused in imbalance in SOD/CAT ratio, suggesting that vitamin A exerts differential effects on the regulation of SOD and CAT activities, which probably contributes to the pro-oxidant effects observed here.

4. Discussion

Vitamin A is known to influence the maturation and differentiation of the lungs during development by affecting different physiological functions of lung cells through modulation of retinoic acid-responsive genes. Type II alveolar cells are especially prepared to synthesize and secrete lung surfactant protein, and retinoic acid was observed to modulate the expression of such protein in fetal lungs [27]. Deficient vitamin A intake was also observed to induce an emphysematous condition in lungs, reducing the content of lung elastin and decreasing type II pneumocyte synthesis of surfactant [28]. Vitamin A is known to preserve and maintain the integrity of the lung epithelium [29]; retinoids have been shown to enhance alveolar septation in neonatal rats and mice [30], and in some cases to improve lung repair after injury in adults [31].

Vitamin A supplementation to human populations considered at elevated risk to lung cancer was nevertheless observed to increase the incidence and mortality of such neoplasia [32], indicating the complexity of vitamin in lung function. Different studies examined the modulation of oxidative parameters in lungs by vitamin A. Diffuse lung disease (DLD) pathogenesis may partly involve oxidative stress driven by an imbalance between oxidant production and antioxidant defenses, since pro-oxidant conditions may promote DLD [33]. Although different studies have explored the potential pro- or antioxidant effects of vitamin A on lung function in animal models, few focused on potential harmful effects on neonates. Considering the role of vitamin A on lung development, the effect of vitamin A supplementation on oxidative stress-related parameters in neonate lung needs to be better understood.

Here, we observed that maternal vitamin A supplementation induced a pro-oxidative state in neonatal lungs that was vitamin A-dose dependent. Vitamin A supplementation increased lipid peroxidation and protein carbonylation levels, and decreased protein thiol content. Furthermore, we observed that vitamin A supplementation to mothers induced a increase on CAT and SOD activities of rat offspring lungs. It is known that vitamin A auto-oxidation *in vitro* increases superoxide anion production in cell cultures [34,35]. Previous studies showed that vitamin A leads to impairment on electron transfer system, thus increasing the rate of superoxide production. This effect was reported either in isolated mitochondria incubated with retinol or in mitochondria isolated from vitamin A supplemented rats [36,37]. Here, we observed that vitamin A supplementation caused an increase in SOD activity, suggesting that superoxide production is enhanced. Taken together, these results indicate a possible exacerbation in superoxide production in this experimental model of supplementation. Increased superoxide production may stimulate SOD activity due to the substrate allosteric activation [26]. In this regard, impairment between the concerted SOD and CAT detoxifying activities may take place, as can be observed in the imbalanced SOD/CAT ratio.

There are many possible deleterious consequences from imbalanced SOD/CAT activities. Increased SOD activity may result in an enhanced formation of H_2O_2 because its production from superoxide dismutation by SOD is also enhanced. An excess of H_2O_2 facilitates the production of the highly reactive and toxic hydroxyl radical ($OH^•$) through a reaction with iron or copper (Fenton chemistry). Van Helden et al. [36] showed that β-carotene and its metabolites (vitamin A, retinal, and retinoic acid) were able to increase $OH^•$ formation in a system containing iron and H_2O_2 through the Fenton reaction. *In vitro* experiments show that either β-carotene or retinol is able to increase cellular iron uptake, thus increasing iron availability in the cytosol to react with H_2O_2 [38,39]. In addition, H_2O_2 is relatively stable and able to diffuse into the nucleus, where it can react with DNA-associated transition metals to form $OH^•$. Oxidatively damaged DNA is known to be pro-mutagenic and is therefore an important marker for carcinogenesis. Previously, we showed that retinol increases iron uptake and induces damage and mutagenesis in the DNA of Sertoli cells through iron-dependent Fenton chemistry [38]. Other authors also observed that vitamin A was able to induce DNA damage [35].

The increase in CAT activity may be associated with the increase of SOD activity and, in turn H_2O_2 formation. Some studies related a conformational change of CAT to (compound II) during exposure to its own substrate H_2O_2 decreases CAT proteasomic degradation and increases its enzymatic activity [40,41]. An imbalance in the SOD/CAT ratio is thought to facilitate oxidative-dependent alterations in the cellular environment. Thus, impaired SOD/CAT is likely to culminate in increased oxidative damage to biomolecules. The basal or physiological SOD/CAT ratio varies according to the tissues, depending on specific-tissue factors such as production of superoxide and H_2O_2 in the electron transport chain, peroxisomes,

and other intrinsic oxidative processes as well. Many studies have shown that an imbalance between SOD and CAT activities is responsible for increasing oxidative stress in biological systems [8,10,42], and this is consistent with our results with maternal vitamin A supplementation in neonatal lungs.

We found that the level of lipid peroxidation increased with vitamin A supplementation. Lipid peroxidation is one of the major sources of free radical-mediated injury that directly damages membranes and generates a number of toxic secondary products. This may involve the interaction of oxygen-derived free radicals with polyunsaturated fatty acids and finally results in a variety of highly reactive electrophilic aldehydes that are capable of easily attaching covalently to proteins by forming adducts with cysteine, lysine, or histidine residues [43]. A large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox signaling pathways depends on the redox properties of the thiol group present both in protein and in low-molecular-weight molecules. Numerous cell signaling proteins are regulated by alterations of the protein thiol groups [44]. This signaling involves cell proliferation, differentiation, and death. Most protein damage is irreparable and may lead to a wide range of downstream functional consequences, such as protein dimerization or aggregation; unfolding or conformational changes to expose more hydrophobic residues to an aqueous environment; loss of structural or functional activity; alterations in cellular handling/turnover; effects on gene regulation and expression; modulation of cell signaling, induction of apoptosis and necrosis [45]. Therefore, an increase of protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in a widespread cellular dysfunction.

Additionally, increased oxidative damage to proteins might result in increased free iron, because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state [46,47]. We observed that vitamin A supplementation increases protein carbonylation of manner dose dependent. In addition, protein thiol levels were decreased with vitamin A supplementation at dose of 500 IU/kg of vitamin A. These effects are also observed in pathological conditions that lead to DLD or lung cancer [48].

In summary, we for the first time show that maternal vitamin A supplementation during pregnancy and lactation increases oxidative stress parameters of neonatal lungs in the rat. Furthermore, these pro-oxidant effects here observed may be involved in the onset of lung diseases associated to redox dysfunctions and free radical-induced damage to biomolecules, such as lung cancer, lung fibrosis, and asthma, and more precautions should be taken when vitamin supplements are prescribed, mainly when administered to children and pregnancy, for therapeutic or preventive interventions.

Conflicts of Interest

None.

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ANEXO II

"L-NAME co-treatment prevent oxidative damage in the lung of adult Wistar rats treated with vitamin A supplementation"

Matheus Augusto de Bittencourt Pasquali, Marcos Roberto de Oliveira, Marco Antônio de Bastiani, Ricardo Fagundes da Rocha, Carlos Eduardo Schnorr, Juciano Gasparotto, Daniel Pens Gelain, José Cláudio Fonseca Moreira

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L-NAME co-treatment prevent oxidative damage in the lung of adult Wistar rats treated with vitamin A supplementation

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Based on the fact that vitamin A in clinical doses is a potent pro-oxidant agent to the lungs, we investigated here the role of nitric oxide (NO·) in the disturbances affecting the lung redox environment in vitamin A-treated rats (retinol palmitate, doses of 1000–9000 IU·kg^{-1}·day^{-1}) for 28 days. Lung mitochondrial function and redox parameters, such as lipid peroxidation, protein carbonylation and the level of 3-nytrotyrosine, were quantified. We observed, for the first time, that vitamin A supplementation increases the levels of 3-nytrotyrosine in rat lung mitochondria. To determine whether nitric oxide (NO •) or its derivatives such as peroxynitrite (ONOO-) was involved in this damage, animals were co-treated with the nitric oxide synthase inhibitor L-NAME (30 mg·kg^{-1}, four times a week), and we analysed if this treatment prevented (or minimized) the biochemical disturbances resulting from vitamin A supplementation. We observed that L-NAME inhibited some effects caused by vitamin A supplementation. Nonetheless, L-NAME was not able to reverse completely the negative effects triggered by vitamin A supplementation, indicating that other factors rather than only NO• or ONOO- exert a prominent role in mediating the redox effects in the lung of rats that received vitamin A supplementation. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—vitamin A; redox environment; L-NAME; lung; mitochondria impairment

INTRODUCTION

In the past few years, vitamin A (retinol) and its metabolites (retinoids) have been frequently suggested to be important antioxidants for tissues such as lungs, liver and heart.[1,2] Additionally, vitamin A therapy (administered mainly as retinol palmitate) at moderate to high doses (30 000–300 000 IU·day^{-1}) is applied to patients suffering from dermatological disturbances and several types of cancer.[3,4] In the lungs, vitamin A is required for fetal development and alveolar septation. During the postnatal period, it participates in the process of tracheal and bronchopulmonary tree formation.[5,6] However, some authors observed that vitamin A may induce toxic effects to different cell types. Daily intervention with β-carotene (30 mg) combined with retinyl palmitate (25 000 IU·kg^{-1}) increased the incidence of lung cancer and colorectal cancer in smokers and asbestos-exposed male individuals.[7–10] Experimental data also associating increased mortality rates with vitamin A supplementation caused concern among public health researchers. However, the mechanisms by which vitamin A elicits such effects are still not clear.

Interestingly, although it is known that vitamin A and retinoids are redox-active molecules, vitamin A has been demonstrated as a pro-oxidant agent '*in vitro*' and '*in vivo*'.[11–15] Previously, we have observed that vitamin A supplementation at clinical doses induced a pro-oxidant effect in the lung of adult Wistar rats under a 28-day oral treatment.[16–18] We showed that vitamin A induces protein carbonylation and lipid peroxidation and that antioxidant enzyme activity is modulated by vitamin A. These pro-oxidant effects of vitamin A supplementation can lead to impaired cell signaling and also induce malignant cell proliferation.[11] In addition, vitamin A directly induces overproduction of superoxide anion (O_2•-) '*in vitro*', resulting in oxidative DNA damage.[19]

Oxidative stress is generated when the production of reactive oxygen species and/or reactive nitrogen species overcomes the cellular antioxidant defense systems. Oxidative stress may cause structural changes and/or degradation of nucleic acids, proteins, and lipids. Increased oxidative stress is a significant part of the pathogenesis of lung cancer and is observed also in lung diseases such as asthma and chronic obstructive pulmonary disease and parenchymal lung diseases (e.g. idiopathic pulmonary fibrosis and lung granulomatous diseases).[20] Reactive oxygen species/reactive nitrogen species may alter the remodeling of extracellular matrix, apoptosis signal and mitochondrial

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respiration inside cells of lungs.[21,22] In addition, oxidative stress may impair the maintenance of surfactant and the antiprotease screen and the immune modulation in the lung.[22]

On the basis that vitamin A supplementation at clinical levels impaired the redox environment in several experimental models and that few reports show the consequences of vitamin A therapy at moderate to high levels regarding lung homeostasis, we investigated here the role of nitric oxide (NO·) in the disturbances affecting the lung redox environment in vitamin A-treated rats. NO· is produced physiologically and mediates, for example, neurotransmission and vascular relaxation.[23] Furthermore, NO· is a free radical whose reactivity may lead to pro-oxidant insults in diverse biological systems directly or indirectly by generating the highly oxidizing species peroxynitrite ($ONOO^-$).[23]

We treated Wistar rats for 28 days with vitamin A (retinol palmitate) at 1000, 2500, 4500 or 9000 $IU{\cdot}kg^{-1}{\cdot}day^{-1}$ and/or L-nitro-arginine methyl ester (L-NAME, an inhibitor of nitric oxide synthase) at 30 $mg{\cdot}kg^{-1}$ in this protocol. We analysed the redox effects of these treatments in the lungs of the rats. As mentioned above, the doses investigated here belong to a therapeutic range usually applied in the treatment of dermatological and oncogenic pathologies.[24]

MATERIALS AND METHODS

Animals

Adult male Wistar rats (290–320 g; 90 days old) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light-dark cycle (7:00–19:00h), at a temperature-controlled colony room (23 ± 1°C). These conditions were maintained constant throughout the experiments. We used 10 animals per group. For the study, 100 animals were used in total. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996). Our research protocol was approved by the ethical committee for animal experimentation of the Federal University of Rio Grande do Sul.

Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Antibody to 3-nitrotyrosine was purchased from Calbiochem, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily, and solutions were kept from light.

Treatment

The animals were treated with retinol palmitate (vitamin A) once a day for 28 days. The treatments were carried out at night (i.e. when the animals are more active and consume a greater amount of food) to ensure maximum vitamin A absorption because this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0·15 $mol{\cdot}L^{-1}$ of saline), 1000, 2500, 4500, or 9000 $IU{\cdot}kg^{-1}$ of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0·6 mL. These vitamin A doses are very similar to those applied to subjects suffering from some types of cancer (mainly leukemia), dermatological disturbances and immunodeficiency.[24] Also, individuals interested in preventing aging progress frequently ingest vitamin A at similar doses inadvertently.[25] Such doses were maintained at values near those that are observed being applied in human therapies because vitamin A metabolism in rats is not significantly different from humans, as previously reported.[26] L-NAME at 30 $mg{\cdot}kg^{-1}$ was dissolved in sterile saline (0·15 $mol{\cdot}L^{-1}$) and i.p. administered to the animals four times a week. Adequate measures were taken to minimize pain or discomfort.

Thiobarbituric acid-reactive species

As an index of lipid peroxidation, we measured the formation of thiobarbituric acid-reactive species (TBARS) during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described. In brief, the samples were mixed with 0·6 ml of 10% trichloroacetic acid and 0·5 ml of 0·67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined using absorbance in a spectrophotometer at 532 nm. Results are expressed as nmol of TBARS/mg of protein.[27,28]

Measurement the levels of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described. In brief, proteins were precipitated by addition of 20% trichloroacetic acid and redissolved in dinitrophenylhydrazine, and the absorbance was read in a spectrophotometer at 370 nm. Results are expressed as nmol of carbonyl/mg of protein.[29]

Measurement of protein thiol content

Protein thiol content in samples was analysed to estimate oxidative alterations in proteins. In brief, an aliquot was diluted in 0·1% sodium dodecyl sulfate, 0·01 $mol{\cdot}l^{-1}$ of 5,50-dithionitrobis(2-nitrobenzoic acid) in ethanol was added, and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min. This experiment also is able to measure non-protein thiol content, in this way, total thiol content. Results are expressed as mmol of SH/mg of protein.[30]

Antioxidant enzyme activities

Superoxide dismutase (EC 1·15·1·1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described, and the results are expressed as units of SOD/mg of protein.[31]Catalase (EC 1·11·1·6) (CAT) activity was assayed by measuring

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the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm, and the results are expressed as units of CAT/mg of protein. Bubble formation in oxygen generation by CAT activity was monitored and did not interfere with measurement of CAT activities in the linear range used to measure CAT activity.[16] A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A treatment on these two oxidant detoxifying enzymes that work in sequence converting superoxide anion to water. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.[32]

Oxidative parameters in submitochondrial particles

Briefly, to obtain submitochondrial particles (SMPs), lungs were dissected and homogenized in 230 mmol·l^{-1} of mannitol, 70 mmol·l^{-1} of sucrose, 10 mmol·l^{-1} of Tris-HCl and 1 mmol·l^{-1} of ethylenediaminetetraacetic acid (EDTA; pH 7·4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution also was washed (twice) with 140 mmol·l^{-1} of KCl and 20 mmol·l^{-1} of Tris-HCl (pH 7·4) to ensure Mn–SOD release from mitochondria. To quantify superoxide radical anion ($O_2^{\cdot-}$) production, SMP was incubated in reaction medium consisted of 230 mmol·l^{-1} of mannitol, 70 mmol·l^{-1} of sucrose, 10 mmol·l^{-1} of HEPES-KOH (pH 7·4), 4·2 mmol·l^{-1} of succinate, 0·5 mmol·l^{-1} of KH_2PO_4, 0·1 μmol·l^{-1} of catalase and 1 mmol·l^{-1} of epinephrine, and the increase in the absorbance (autooxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described.[33] As a marker of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction.[27] The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine.[29] Protein thiol content in lung SMP samples were determined as described above.[30]

Mitochondrial electron transfer chain activity

To obtain SMP from lung in order to assess mitochondrial electron transfer chain (METC) activity, we have homogenized the tissue in 250 mmol·l^{-1} of sucrose, 2 mmol·l^{-1} of EDTA, 10 mmol·l^{-1} of Tris, pH 7·4 and 50 IU·ml^{-1} of heparin buffer. The samples were centrifuged 1000 *g*, and the supernatants were collected. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.[12]

Complex I-CoQ-III activity

Complex I-CoQ-III activity was determined by following the increase in absorbance because of reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in$ = 19·1 mmol·l^{-1} cm^{-1}). The reaction buffer contained 20 mmol·l^{-1} of potassium phosphate, pH 8·0, 2·0 mmol·l^{-1} of KCN, 10 μmol·l^{-1} of EDTA, 50 μmol·l^{-1} cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μmol·l^{-1} of NADH and was monitored at 30 °C for 3 min before the addition of 10 μmol·l^{-1} of rotenone, after which, the activity was monitored for an additional 3 min. Complex I-III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity.[34]

Complex II and succinate dehydrogenase activities

Complex II (succinate-DCPIP-oxidoreductase) activity was measured by following the decrease in absorbance because of the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm with 700 nm as reference wavelength ($\in$ = 19·1 mmol·l^{-1} cm^{-1}). The reaction mixture consisting of 40 mmol·l^{-1} of potassium phosphate, pH 7·4, 16 mmol·l^{-1} of succinate and 8·0 μmol·l^{-1} of DCPIP was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4·0 mmol·l^{-1} of sodium azide and 7·0 μmol·l^{-1} of rotenone were added, and the reaction was started by addition of 40 μmol·l^{-1} of DCPIP and was monitored for 5 min at 30 °C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mmol·l^{-1} of phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength.[35]

Complex II-CoQ-III activity

Complex II-CoQ-III activity was measured by following the increase in absorbance because of the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\in$ = 21 m mol·l^{-1} cm^{-1}). The reaction mixture consisting of 40 mmol·l^{-1} of potassium phosphate, pH 7·4, and 16 mmol·l^{-1} of succinate was preincubated with 50–100 μg of supernatant protein at 30 °C for 30 min. Subsequently, 4·0 mmol·l^{-1} of sodium azide and 7·0 μmol·l^{-1} of rotenone were added, and the reaction started by the addition of 0·6 μg·ml^{-1} of cytochrome *c* and monitored for 5 min at 30 °C.[35]

Indirect enzyme-linked immunosorbent assay to 3-nitrotyrosine

Indirect enzyme-linked immunosorbent assay was performed to analyse changes in the content of 3-nitrotyrosine (3-NT) by utilizing a polyclonal antibody to 3-nitrotyrosine diluted 1:5000 in phosphate-buffered saline (PBS), pH 7·4, with 5% albumin. Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:5 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0·05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (four times), a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3,3,5,5-tetramethylbenzidine, 1:1, v/v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups.

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Statistical analyses

Biochemical results are expressed as means ± SEM; *P* values of <0·05 were considered significant. Differences in experimental groups were determined using *T* test.

RESULTS

We observed increased lipid peroxidation levels in the lungs of the rats that received vitamin A supplementation at 1000, 2500, 4500 or 9000 IU·kg^{-1} ($P < 0·05$) (Figure 1A). This increase in lipid peroxidation levels is dose dependent. This result is in agreement with our previous results.[16,17]

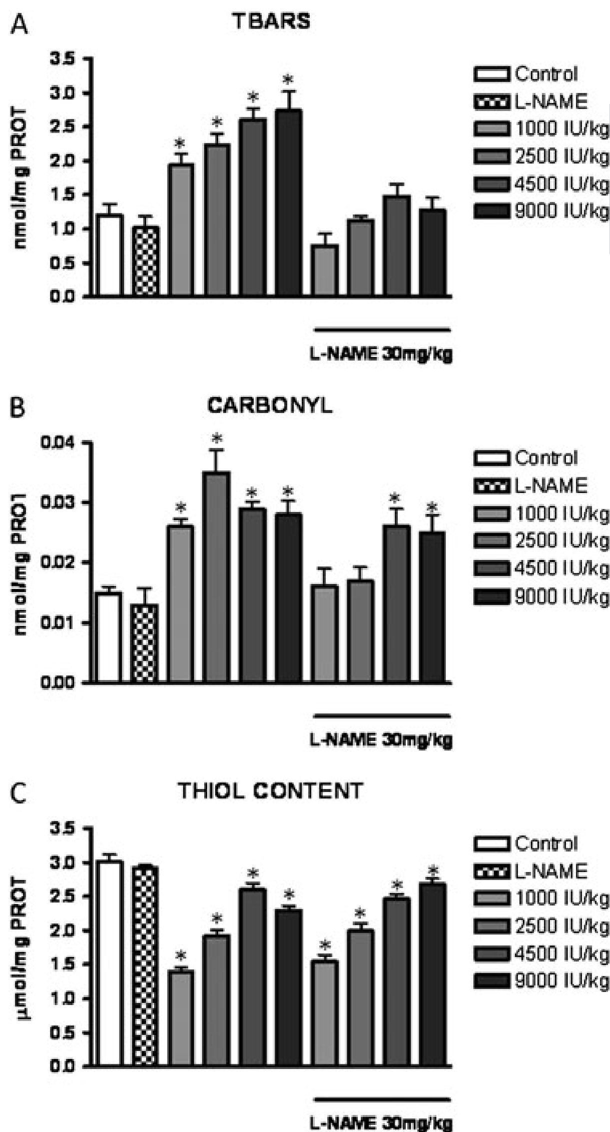

Figure 1. Effects of vitamin A supplementation and/or L-NAME administration on lung lipid peroxidation (TBARS) (A), protein carbonylation (CARBONYL) (B) and thiol content (C). Data are mean ± SEM ($n = 10$ per group). $^{*}P < 0·05$ (vitamin A with or without L-NAME versus control group *T* test)

The co-treatment with L-NAME reversed the effect caused by vitamin A supplementation at all doses tested ($P < 0.05$) (Figure 1A). Besides, lung protein carbonylation levels increased ($p < 0·05$) at all doses tested (Figure 1B) as previously observed[16,17]; vitamin A supplementation at 1000 IU·kg^{-1} increased lung carbonyl levels by twofold and higher doses presented a similar effect. We also observed that L-NAME inhibited the effect of vitamin A on protein carbonylation at doses of 1000 and 2500 IU·kg^{-1}. At doses of 4500 and 9000 IU·kg^{-1}, the co-treatment with L-NAME was not able to attenuate the effect induced by vitamin A supplementation. All doses also were observed to induce a significant decrease in lung protein thiol content as compared with control group, confirming that vitamin A supplementation exerted a pro-oxidant effect on lung proteins. Interestingly, the doses of 1000 and 2500 IU·kg^{-1} presented the most pronounced effect on lung thiol levels, whereas the dose of 4500 IU·kg^{-1} had a milder effect (Figure 1C). At 9000 IU·kg^{-1}, the effect was not statistically different from the decrease in thiol content observed at 1000 and 2500 IU·kg^{-1}. Co-treatment with L-NAME was not able to alter the effect caused by vitamin A supplementation in lung protein thiol content.

We next investigated the activity of the antioxidant enzymes SOD and CAT in the lungs of rats that received vitamin A supplementation. SOD activity was increased ($P < 0·05$) in the lung of the rats that received vitamin A at 4500 or 9000 IU·kg^{-1} (Figure 2A) as previously observed.[16,17] On the other hand, vitamin A supplementation induced a significant decrease ($P < 0·05$) in lung CAT activity at doses 2500, 4500 and 9000 IU·kg^{-1} (Figure 2B). Co-treatment with L-NAME was not effective in attenuating the changes induced by vitamin A on SOD activity. However, L-NAME reversed the effect of vitamin A in CAT activity. Besides, as depicted in Figure 2C, vitamin A supplementation at any dose induced an imbalance ($P < 0·05$) in SOD/CAT activity ratio. Altogether, these data indicate that vitamin A induces differential effects on the regulation of activity of CAT and SOD, which probably contributes to the pro-oxidant status of lung tissue reported in this work.

As shown in Figure 3A, vitamin A supplementation at 2500, 4500 or 9000 IU·kg^{-1} induced an increase in the production of O_2·- in lung SMP ($P < 0·05$). Lipid peroxidation levels were increased in SMP isolated from the lung of rats that received vitamin A supplementation at 9000 IU·kg^{-1} ($P < 0·05$) (Figure 3B). Additionally, vitamin A supplementation at 2500, 4500 or 9000 IU·kg^{-1} increased the levels of protein carbonylation in lung SMP ($P < 0·05$) (Figure 3C). Interestingly, mitochondrial protein sulfhydryl content did not differ among groups in this experimental model (Figure 3D). The co-treatment with L-NAME did not reverse the effects caused by vitamin A supplementation in lung mitochondria.

We observed an increased content of 3-NT in SMP isolated from lungs of vitamin A-treated rats at 4500 or 9000 IU·kg^{-1} ($P < 0·05$; Figure 4A). The co-treatment with L-NAME reversed the effect of vitamin A at 4500 IU·kg^{-1}.

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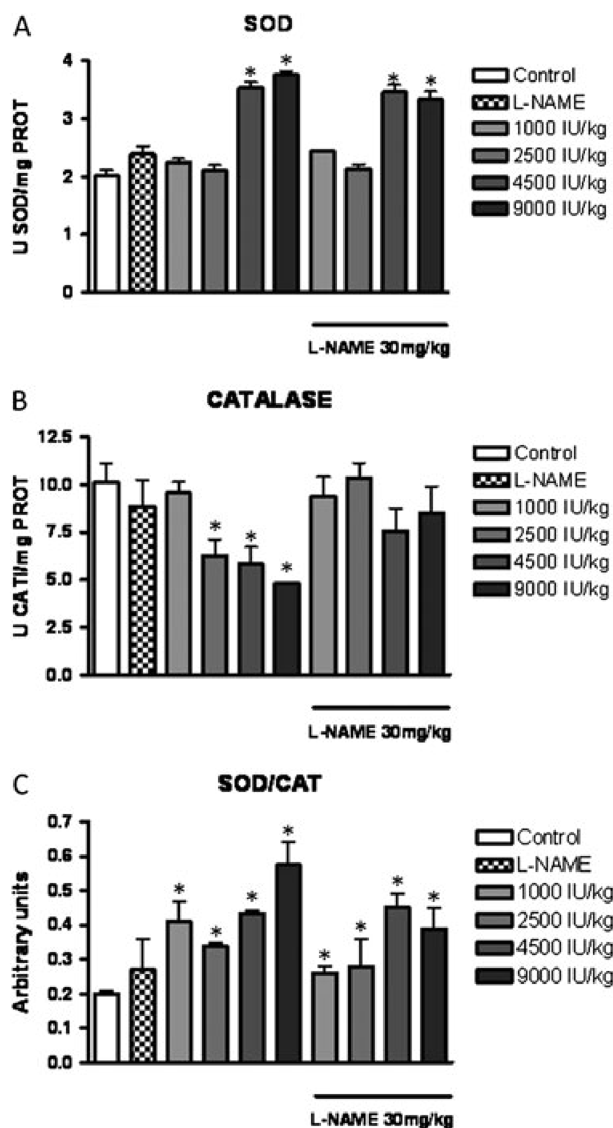

Figure 2. Effects of vitamin A supplementation and/or L-NAME administration on lung SOD activity (A), CAT activity (B) and SOD/CAT ratio (C). Data are mean ± SEM ($n = 10$ per group). $^{*}P < 0{\cdot}05$ (vitamin A with or without L-NAME versus control group *T* test)

At a dose of $9000\,IU{\cdot}kg^{-1}$ of vitamin A, L-NAME was unable to reverse 3-NT formation. We also monitored MECT activity and observed an increase in complex I-III enzyme activity in the lung of the rats that received vitamin A at $9000\,IU{\cdot}kg^{-1}$ ($P < 0{\cdot}05$; Figure 4B).The same effect was observed in complex II-III, SDH, and complex II enzyme activity (Figure 4C, D, and E respectively). L-NAME was unable to reverse the effects of vitamin A supplementation on MECT activity.

DISCUSSION

Vitamin A is known to influence the maturation and differentiation of the lungs during development by affecting different physiological functions of lung cells through modulation of retinoic acid-responsive genes. Type II alveolar cells are especially prepared to synthesize and secrete lung surfactant protein, and retinoic acid was observed to modulate the expression of such protein in fetal lungs.[36,37] Deficient vitamin A intake also was observed to induce an emphysematous condition in lungs, reducing the content of lung elastin and decreasing type II pneumocyte synthesis of surfactant.[38,39] However, vitamin A supplementation to human populations considered at elevated risk to lung cancer was nevertheless observed to increase the incidence and mortality in such neoplasia, indicating the complexity of vitamin A in lung function.[7,9]

In the present work, we evaluated the effects of vitamin A supplementation on rat lung, regarding oxidative/nitrosative stress and mitochondrial function. We show that vitamin A supplementation at doses commonly used therapeutically induces lung nitrosative stress and an imbalanced METC activity. Furthermore, we observed that co-treatment with L-NAME, which is a nitric oxide synthase inhibitor, reversed some effects caused by vitamin A supplementation, thus demonstrating a possible involvement of $ONOO^{-}$ in such effects. Although oxidative stress may be induced in the cellular environment through exposition to some pro-oxidant compounds, METC activity is responsible for about 20% of total $O_2{\cdot}^{-}$ production physiologically.[40] Mitochondrial dysfunction consequently increases the rate of free radical production to dangerous levels, which may culminate in oxidative damage to biomolecules and protein function loss.

Once it was already observed in the previous work,[16,17] here, we observed that vitamin A supplementation induced a pro-oxidative state. Vitamin A treatment increases lipid peroxidation in both lungs and SMP. The level of protein carbonylation also increases in both lung tissue and in SMP. We also observed a decrease in protein thiol content in lung tissue suggesting an oxidative stress situation. Interestingly, vitamin A supplementation did not alter protein thiol content in SMP. The activities of CAT and SOD also were modulated by vitamin A supplementation and co-treatment with L-NAME was able to partially reverse the effect caused by vitamin A supplementation, therefore demonstrating the possible involvement of the $ONOO^{-}$ in the pro-oxidant effects of vitamin A.

Increased protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in widespread cellular dysfunctions.[41] Additionally, increased oxidative damage to proteins might result in increased free iron because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state by facilitating the production of hydroxyl radical (${\cdot}OH$), a highly oxidant molecule, through reaction with iron or copper (Fenton chemistry).[42,43] Van Helden *et al*. showed that both vitamin A, retinal, and retinoic acid were able to increase ${\cdot}OH$ formation from a system containing iron and hydrogen peroxide (H_2O_2) through the Fenton reaction.[44,45]

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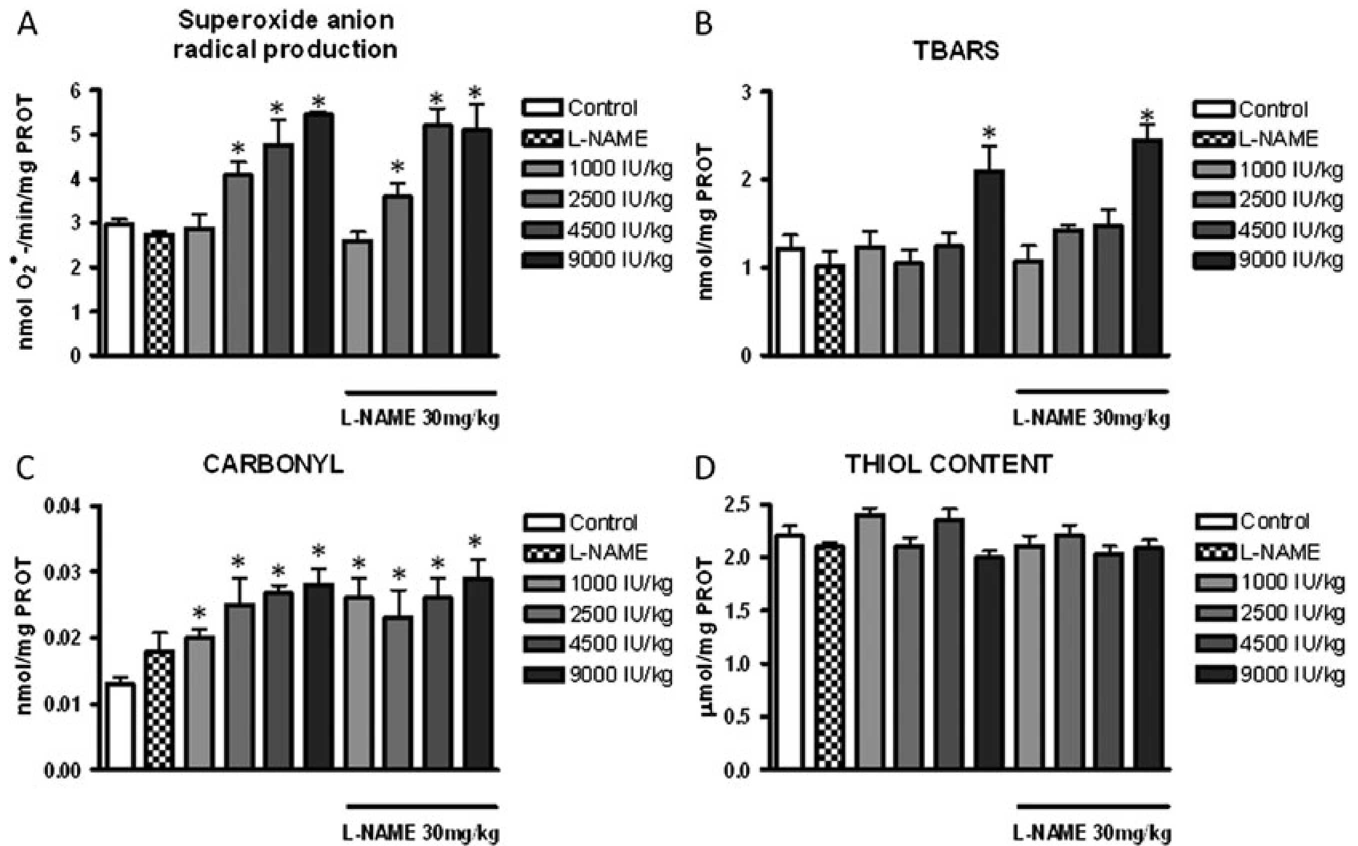

Figure 3. Effects of vitamin A supplementation and/or L-NAME administration on lung submitochondrial particles $O_2^{\bullet-}$ production (A), lipid peroxidation (TBARS) (B), carbonylation (C) and thiol content (D). Data are mean ± SEM ($n = 10$ per group). $^{*}P < 0{\cdot}05$ (vitamin A with or without L-NAME versus control group *T* test)

The increase in •OH also is associated with increase in lipid peroxidation.[46] In the present work, we observed that lipid peroxidation in the lung tissue and SMP of rats treated with vitamin A were increased. Products of lipid peroxidation are toxic; lipid hydroperoxides can directly inhibit enzymes. Moreover, increased oxidative damage may culminate in pathological conditions that lead to idiopathic pulmonary fibrosis.[20]

We observed here a mitochondrial impairment in the lungs of vitamin A supplemented animals. We found increased complex I–III enzyme activity, and the same effect was observed in complex II–III, SDH and complex II enzyme activity. Moreover, we observed increased O2•- production. Levels of lipid peroxidation and protein carbonylation also were enhanced in SMP. These data suggest that MECT impairment may lead to enhanced formation of O_2•- because of partial reduction of oxygen at complex IV, leading to mitochondrial oxidative damage.[33] Moreover, the excess of O_2•- may react with NO•, leading to formation of ONOO‾. The imbalance on nitrogen reactive species is known as nitrosative stress.[47,48] We then decided to evaluate the content of 3-NT in lung SMP. The 3-NT is produced by the attack that protein tyrosil residues undergo in front of ONOO‾, which is formed by the reaction between O_2•- and the radical NO•.[48]In the present work, an increase in 3-nitrotyrosine content was detected. Interestingly, the co-treatment with L-NAME did not reverse the effects caused by vitamin A supplementation in SMP of the lung. Then, L-NAME was not effective in preventing the perturbation triggered by vitamin A supplementation on lung mitochondria.

In summary, we for the first time show that vitamin A supplementation at doses commonly used therapeutically induced lung nitrosative stress, an imbalanced increase in the METC activity in the rat lungs. Furthermore, these pro-oxidant effects may be involved in the onset of lung diseases associated to redox dysfunctions and free radical-induced damage to biomolecules, such as lung cancer, lung fibrosis and asthma. Moreover, it is not careful to rule out a role for NO· in the disturbances triggered by vitamin A, since L-NAME was partially effective in preventing some effects observed in the lungs of vitamin A-treated rats. More analyses are necessary to elucidate the mechanisms by which vitamin A affects lungs homeostasis. So we suggest that more precautions should be taken when vitamin supplements are prescribed, mainly when administered to children and pregnant women, for therapeutic or preventive interventions.

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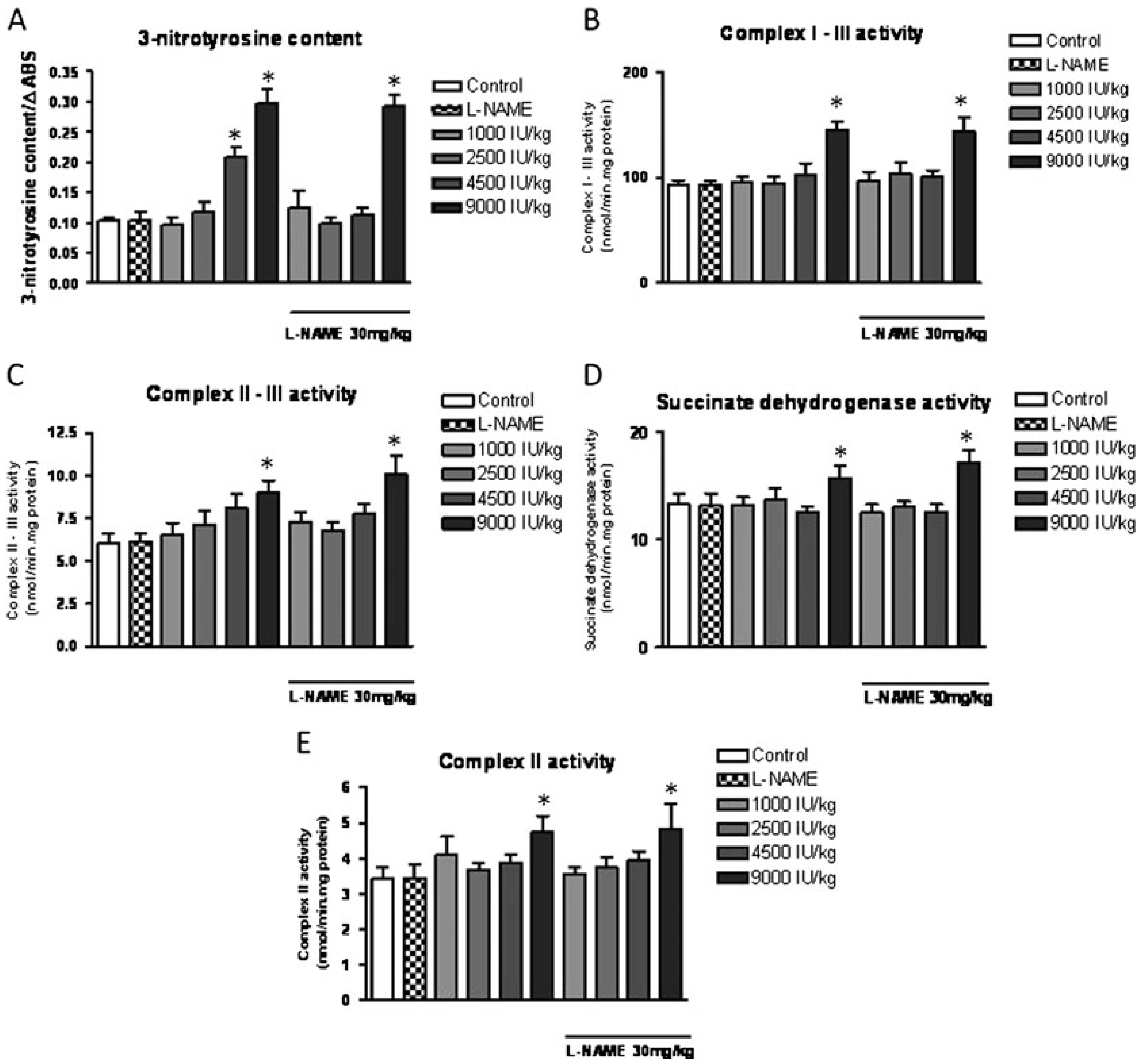

Figure 4. Effects of vitamin A supplementation and/or L-NAME administration on rat lung 3-nitrotyrosine mitochondrial content (A), complex I–III acitivity (B), complex II–III activity (C), succinate dehydrogenase activity (D) and complex II activity (E). Data are mean ± SEM ($n = 10$ per group). $^{*}P < 0.05$ (vitamin A with or without L-NAME versus control group T test)

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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