UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
PPG – CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Inosina extracelular como intermediária na sinalização do TNF-α em células de Sertóli em cultura.

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Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre pelo PPG – Ciências Biológicas: Bioquímica, Departamento de Bioquímica, ICBS, UFRGS.

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Porto Alegre, fevereiro de 2004.

Agradecimentos

Agradeço, em primeiro lugar, à minha família, que sempre me apoiou e incentivou a continuar buscando meus objetivos. Não poderia deixar de citar aqui minha namorada, Paolinha, amor da minha vida, e sua família, os quais considero também minha família.

Agradeço à todos os colegas, que durante o período em que estive trabalhando no Laboratório 23, deram-me o prazer de compartilhar de sua companhia e parceria no trabalho: Émerson, Glória, Dona Lia, Fernanda, Marcelo, Gisele, Fabiano, Vanessa e Andrei. Em especial, ao grande amigo e parceiro Daniel Pens Gelain, vulgo Geléia, que me acompanhou e ajudou ao longo de todo o tempo em que estive no Departamento de Bioquímica.

Agradeço aos colegas dos Laboratórios 21 (Eduardo, Cláudia e Ana) e 25 (Michael, Felipe, Fábio, Fernanda, Manoela, Evandro, Mário, Ramatis, Martina, Amâncio, Guilherme, Márcio, Marcos, Mauro), pelas diversas horas produtivas ou ociosas, no entanto, sempre agradáveis, que passamos juntos ao longo destes vários anos em que estive no Departamento.

Agradeço ao professor e amigo José Cláudio Fonseca Moreira, o qual me trouxe para este Departamento.

Agradeço à minha amiga e colega de graduação e pós-graduação Ana Paula Horn pela colaboração na execução de grande parte deste trabalho.

Agradeço aos professores e funcionários deste Departamento que tornam possível a sua existência.

Por último, e principalmente, agradeço à minha orientadora, no sentido mais completo que esta palavra pode ter, a "Professora" Elena Aida Bernard, pela confiança em mim depositada ao longo de todo este tempo em que trabalhamos juntos, deste de minha iniciação científica até agora. Durante este tempo aprendi muito, principalmente pelo contínuo incentivo e liberdade para pensar e criar.

Índice

Lista de Abreviaturas

ATP - adenosina trifosfato

ADA - adenosina deaminase

cAMP - adenosina monofosfato cíclico

FSH - hormônio estimulante do folículo

TNF-α - fator de necrose tumoral alfa

TNF-RI - receptor do fator de necrose tumoral alfa I

TNF-RII - receptor do fator de necrose tumoral alfa II

IGFBP3 – proteína ligadora do fator de crescimento semelhante à insulina 3

NO - óxido nítrico

NOS - óxido nítrico sintase

cGMP - guanosina monofosfato cíclico

LPS - lipopolissacarídeo

MAPK – proteína cinase ativada por mitógenos

ERK ½ - proteína cinase regulada por sinal extracelular 1 e 2

SAPK - proteína cinase ativada por estresse

JNKs - c-jun N terminal proteína cinase

Resumo

As purinas extracelulares ATP e adenosina têm sido extensivamente estudadas em diferentes modelos e tipos celulares na modulação de várias respostas fisiológicas e patológicas. No entanto, a inosina extracelular, produto da degradação da adenosina pela Adenosina Deaminase (ADA), foi considerada por muito tempo um simples metabólito inativo. Recentemente, diversos trabalho têm demonstrado que este nucleosídeo possui importante papel na regulação de inúmeros processos. As células de Sertóli são as células somáticas dos túbulos seminíferos, e possuem fundamental importância na espermatogênese. Estas células, expressam diferentes purinoreceptores, estando estes envolvidos na regulação de diversas funções destas células relacionadas ao controle do desenvolvimento das células germinativas. No testículo, o TNF-α é produzido pelas espermátides redondas e pelos macrófagos ativados presentes no espaço intersticial. As células de Sertóli expressam os dois receptores descritos para TNF-α, TNF-RI (p55) e TNF-RII (p75), e diversos trabalhos tem descrito a modulação de diferentes funções destas células por esta citocina, incluindo a modulação da produção de NO e da fosforilação das MAPKs. Recentemente, foi descrita a modulação purinérgica da sinalização por TNF-α, bem como, a atividade ATPásica do receptor TNF-R1. Assim, nesta dissertação, foi estudado o efeito do TNF-α nos níveis das purinas extracelulares, além da possível participação purinérgica na sinalização desta citocina, em células de Sertóli em cultura. O tratamento destas células com TNF-α leva a um rápido aumento (5minutos) da concentração extracelular da inosina, que se prolonga até seis horas de incubação, sem alterar a concentração dos demais nucleotídeos e seus metabólitos. A inosina modula a produção de NO e a fosforilação das MAPKs ERK½ e p38 em células de Sertóli em

cultura, aparentemente, através de diferentes mecanismos, sendo o primeiro efeito independente do receptor para adenosina A1 e o segundo efeito dependente da ativação deste receptor. Além disso, a inosina extracelular está envolvida na modulação da produção de NO e da fosforilação da MAPK ERK½ em células de Sertóli em cultura pelo TNF-α. A inibição do acúmulo de inosina estimulado pelo TNF-α através da incubação com um inibidor da adenosina deaminase cancela o aumento da produção de NO estimulada por esta citocina. Além disso, o bloqueio do receptor para adenosina A1 por antagonistas específicos impede o aumento na fosforilação da ERK½ estimulada por esta citocina. Assim, nesta dissertação, é descrito um papel intermediário da inosina extracelular na sinalização do TNF-α em células de Sertóli em cultura.

CAPÍTULO I

INTRODUÇÃO

1.1 - Introdução

As purinas extracelulares ATP e adenosina têm sido extensivamente estudadas em diferentes modelos e tipos celulares na modulação de várias respostas fisiológicas e patológicas. Estas moléculas regulam diferentes rotas de sinalização através da ativação de receptores de membrana específicos, designados receptores P1 e P2 (Dubyak e El-Moatassim 1993, Burnstock e Williams 2000, Fredholm *et al.* 2001). Os receptores de adenosina, ou P1, respondem principalmente a este nucleosídeo e são divididos em quatro subtipos: A1, A2A, A2B e A3, todos receptores acoplados a proteínas G (Fredholm *et al.* 2001). Os receptores P2, responsivos principalmente a ATP, são divididos em duas famílias: os receptores ionotrópicos P2X e os receptores metabotrópicos, acoplados a proteínas G, P2Y (Dubyak e El-Moatassim 1993, Burnstock e Williams 2000). A inosina extracelular, produto da degradação da adenosina pela Adenosina Deaminase (ADA), foi considerada por muito tempo um simples metabólito inativo. Entretanto, diversos trabalhos têm demonstrado que este nucleosídeo possui importante papel na regulação de inúmeros processos. Em mastócitos, a inosina extracelular aumenta a degranulação (Jin *et at.*1997), levando a uma maior permeabilidade dos vasos sangüíneos de camundongos (Tilley *et al.* 2000). Além disso, a inosina melhora a resposta renal (Fernando *et al.* 1976, De Rougemont *et al.* 1982) em situações de isquemia e diminui a morte causada por estresse oxidativo em macrófagos RAW 264.7 (Virág and Szabó 2000) e pela privação de glicose em células gliais (Haun *et al.* 1996, Jurkowitz *et al.*1998). Este nucleosídeo também melhora o prognóstico em modelos de endotoxemia (Haskó *et al.* 2000) e lesão pulmonar aguda (Liaudet *et al.* 2002) através da redução da produção de citocinas pró-inflamatórias e aumento da produção de citocinas antiinflamatórias.

As células de Sertóli são as células somáticas dos túbulos seminíferos (Figura 1), e possuem fundamental importância na espermatogênese. Estas células fornecem suporte energético e físico às células germinativas, além de mediar a regulação hormonal do processo espermatogênico. As células de Sertóli também formam a barreira hemato-testicular, indispensável à manutenção de um ambiente imunologicamente privilegiado necessário para o desenvolvimento das células germinativas (Jégou e Sharpe 1993). Diferentes purinoreceptores são expressos pelas células de Sertóli, estando estes envolvidos na regulação de diversas funções destas células relacionadas ao controle da espermatogênese, incluindo: receptor de adenosina A1, relacionado com a inibição da acumulação de cAMP estimulada pelo FSH (Monaco e Conti 1986, Rivkees 1994, Monaco *et al.* 1998); receptor P2Y2 responsivo a ATP, que regula o ciclo de fosfatidil-inositol e a mobilização de cálcio, inibindo a cumulação de cAMP (Filippini *et al.* 1994) e aumentando a expressão de γ-glutamil-transpeptidase e a secreção de transferrina (Meroni *et al.* 1998); e receptores ionotrópicos P2X(2,3 e 7) responsivos a ATP, os quais são expressos em diferentes estágios do ciclo do epitélio seminífero (Glass *et al.* 2001). Além disso, previamente foi descrita a atividade de diferentes enzimas extracelulares envolvidas na degradação de ATP até inosina em células de Sertóli (Casali *et al.* 2001), bem como a secreção de nucleotídeos extracelulares e seus metabólitos pelas células do túbulo seminífero, sugerindo uma função na sinalização parácrina para estas moléculas (Gelain *et al.* 2003).

A citocina TNF-α foi inicialmente descrita em células do sistema imune ativadas, através da sua ação citotóxica em diferentes linhagens tumorais. Posteriormente, a produção de TNF-α, bem como sua ação, começou a ser descrita em diferentes tipos

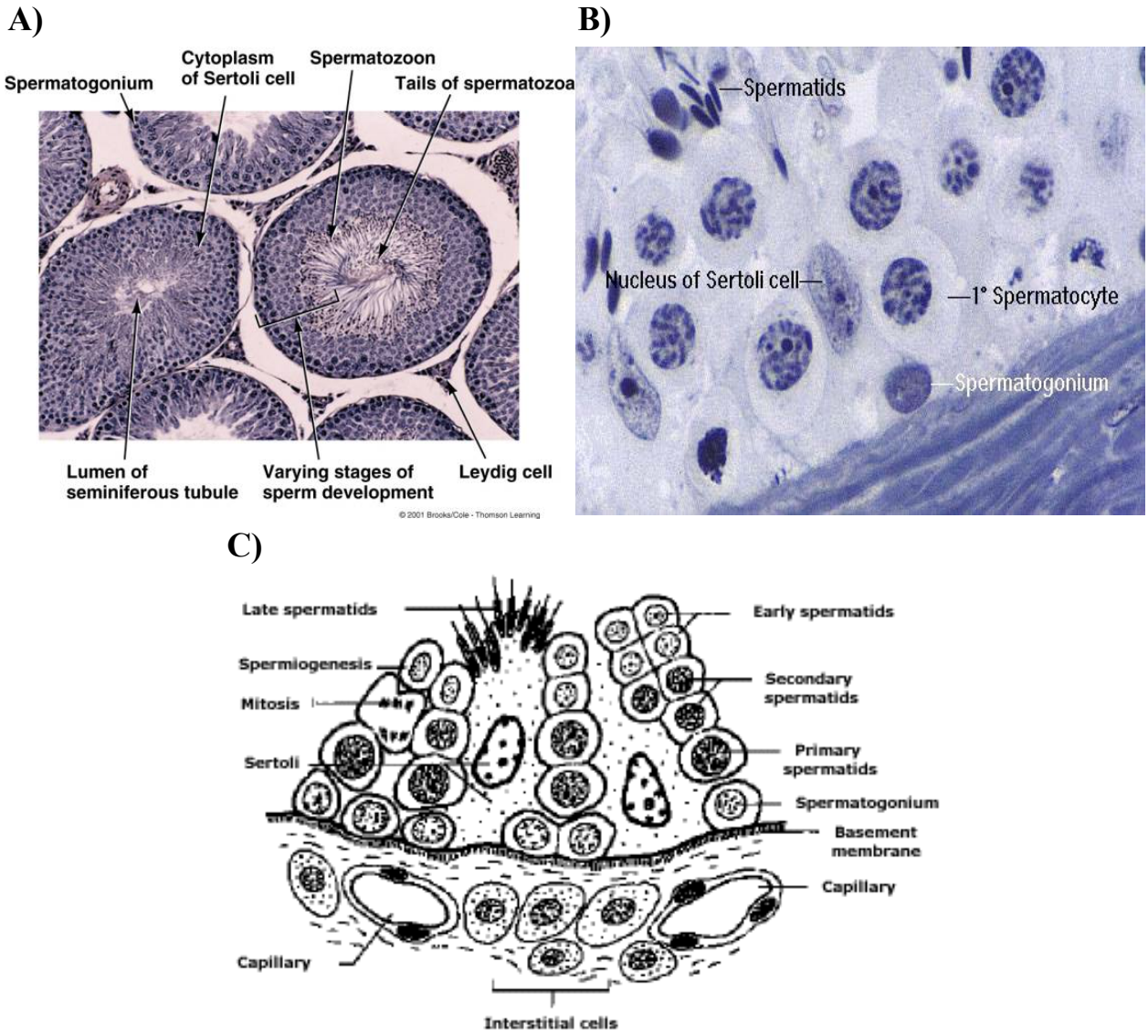

Figura 1: Estrutura dos túbulos seminíferos. A) Secção transversal de túbulos seminíferos mostrando sua estrutura geral, com os diferentes tipos celulares, bem como, o espaço intersticial entre os túbulos. B) Detalhe de uma porção do túbulo seminífero, destacando a célula de Sertóli e diferentes éstágios de desenvolvimento das células germinativas. C) Representação esquemática de uma porção do tubulo seminífero com parte do espaço intersticial.

celulares, ampliando o número de funções desta molécula (Wajant *et al.* 2003). No testículo, esta citocina é produzida pelas espermátides redondas em situações fisiológicas (De *et al.* 1993), entretanto, em situações inflamatórias, macrófagos ativados presentes no espaço intersticial do testículo podem produzir grande quantidade desta citocina (Xiong e Hales 1993, Moore e Hutson 1994). As células de Sertóli expressam os dois receptores descritos para TNF-α, TNF-R1 (p55) e TNF-R2 (p75) (Mauduit *et al.* 1996, De Cesaris *et al.* 1999), e diversos trabalhos tem descrito a modulação de diferentes funções destas células por esta citocina. O TNF-α modula a expressão de interleucina-6 (Riccioli *et al.* 1995), inibina (Magueresse-Battistoni *et al.* 1995), IGFBP-3 (Besset *et al.* 1996) e transferrina (Sigillo *et al.* 1999), bem como a secreção de lactato (Nehar *et al.* 1997). Além disso, o TNF-α modula diferentes ações do FSH (Mauduit *et al.* 1993), o sistema Fas-ligante (Pentikäinen *et al.* 2001) e a dinâmica das junções oclusivas (Siu *et al.* 2003) nas células de Sertóli.

O óxido nítrico (NO) é produzido pelas óxido nítrico sintases (NOS), convertendo arginina em citrulina, por diferentes tipos celulares, estando relacionadas com a regulação de diversos processos fisiológicos e patológicos. São descritas três isoformas de NOS, a neuronal, a endotelial e a induzível, sendo as duas primeiras dependentes de cálcio e constitutivas, enquanto que a induzível é expressa em resposta a diferentes estímulos, como citocinas pró-inflamatórias, e é cálcio-independente. Em células de Sertóli, é descrita a presença das três isoformas (Middendorff *et al.* 1997), estando a produção de óxido nítrico relacionada com a modulação da produção de cGMP e com a regulação de processos relacionados com a dinâmica das junções oclusivas (Lee e Cheng 2003a, 2003b). O TNF-α aumenta a expressão da NOS induzível em células de Sertóli (Lee e Cheng 2003a), além de

aumentar a produção de NO em combinação com interferon-γ e/ou LPS (Stéphan *et al.* 1995, Bauché *et al.* 1998). Adicionalmente, a modulação da produção de NO por purinas extracelulares, incuindo ATP, adenosina e inosina, é descrita em vários tipos celulares (Sperlágh *et al.* 1998, Min *et al.* 2000, Ohtani *et al.* 2000, Liu *et al.* 2002).

O TNF-α também regula a fosforilação das proteínas cinases ativadas por mitógenos (MAPKs) ERK ½ e p38 em células de Sertóli (De Cesaris *et al.* 1998). As MAPKs são serina/treonina cinases envolvidas na regulação do ciclo celular, proliferação, diferenciação e morte celular. A família das MAPKs consiste de três subgrupos principais: as cinases reguladas por sinais extracelulares (ERKs), as proteínas cinases ativadas por estresse (SAPKs) p38 e as SAPKs c-jun N terminal cinases (JNKs) (Schulte e Fredholm 2003). A modulação das MAPKs pela adenosina extracelular através da ativação dos receptores para adenosina A1, A2A, A2B e A3 é bem conhecida (revisado em Schulte e Fredholm 2003). Além disso, o ATP extracelular também é descrito modulando as MAPKs através da ativação de receptores P2 (Sellers *et al.* 2001, Shigemoto-Mogami *et al.* 2001, Bradford e Soltoff 2002, Gendron *et al.* 2003). Entretanto, não é descrita a modulação das MAPKs pela inosina extracellular.

Recentemente, foi descrita a modulação purinérgica da sinalização por TNF-α em neutrófilos (Barnes *et al.* 1995) e astrócitos (Liu *et al.* 2000). Além disso, Miki e Eddy (2002) demonstraram que o receptor para TNF-α TNF-R1 apresenta atividade ATPásica, envolvida na agregação deste receptor em resposta à ativação pela citocina. Desta maneira, a ligação do TNF-α em seu receptor pode levar a uma alteração dos níveis intracelulares de purinas, que através dos transportadores equilibrativos de nucleotídeos/nucleosídeos podem alterar os níveis de purinas extracelulares.

Assim, nesta dissertação, foi estudado o efeito do TNF-α nos níveis das purinas extracelulares em células de Sertóli em cultura. Também foi estudada uma possível participação purinérgica na sinalização por esta citocina na estimulação da produção de NO e no aumento da fosforilação da ERK ½ e p38.

1.2 - Objetivos

- Estudar a ação do TNF-α na concentração dos nucleotídeos extracelulares e de seus metabólitos em células de Sertóli em cultura;

- Estudar a ação da inosina na produção de NO em células de Sertóli em cultura;

- Estudar a participação da inosina extracelular na modulação da produção de NO pelo TNF-α em células de Sertóli em cultura;

- Estudar a ação da inosina extracelular na fosforilação das MAPKs ERK ½ e p38 em células de Sertóli em cultura.

CAPÍTULO 2

Inosina extracelular como mediadora da modulação da produção de óxido nítrico pelo TNF-α em células de Sertóli em Cultura

Manuscrito enviado para publicação na revista

Archives of Biochemistry and Biophysics.

subject area: cell biochemistry

EXTRACELLULAR INOSINE MEDIATES TNF-α INDUCED NITRIC OXIDE PRODUCTION IN SERTOLI CELLS

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short title: Inosine, TNF-α and NO

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Fax number: 55-51-3316.5535/5540

ABSTRACT

Recent reports have described purinergic modulation of tumor necrosis factor-α (TNF-α) signalling in neutrophils and astrocytes. In Sertoli cells both TNF-R1 and TNF-R2 TNF-α receptors are present and this cytokine modulates many functions related to the maintenance of spermatogenesis in these cells. Sertoli cells express distinct purinoreceptors and previous work has shown that these cells secrete extracellular nucleotides and their metabolites. We studied the possible role of extracellular purines in TNF-α signalling in cultured Sertoli cells. This cytokine increased inosine concentration after 30 minutes to 6 hours, with no effect at 24 hours. Both TNF-α and inosine increased nitrite accumulation and nitric oxide synthase activity. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase inhibitor, abolished the TNF-α induced inosine increase, nitrite accumulation and nitric oxide synthase activity. These results suggest that extracellular inosine acts as intermediary in TNF-α stimulated nitric oxide production in cultured Sertoli cells.

Keywords – Sertoli cell, tumor necrosis factor-α, TNF, extracellular purines, inosine, nitric oxide, NO, nitric oxide synthase, NOS, adenosine.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine involved in the regulation of diverse physiological and pathological responses through the activation of TNF-R1 (p55) and TNF-R2 (p75) receptors. The molecular mechanisms of signal transduction by TNF-α receptors involve the recruitment of intracellular adapter proteins that link TNF receptors to many signaling processes [for reviews see 1,2]. In normal conditions, round spermatids are the main producers of TNF-α in the testis [3], where this cytokine exerts a regulatory function on spermatogenesis. However, in inflammatory responses, interstitial macrophages can account for considerable production of this cytokine [4,5]. Sertoli cells, which support germinative cells during spermatogenesis, express both TNF-α receptors [6,7], and this cytokine modulates various activities in these cells, such as lactate production [8], and interleukin-6 [9], inhibin [10], transferrin [11] and insulin–like growth factor binding protein 3 expression [12]. These Sertoli cell functions are strongly related to the maintenance and regulation of spermatogenesis. In addition, TNF-α modulates distinct FSH actions [13], the Fas-ligand system [14] and tight junction dynamics [15] in these cells.

Sertoli cells express various purinoreceptors: A1 adenosine receptors, which are related to the inhibition of FSH-stimulated cAMP accumulation [16-18]; the $P2Y_2$ ATP receptor, which regulates phosphatidyl-inositol turnover and calcium mobilization, inhibiting cAMP accumulation [19] and increasing γ-glutamyl-transpeptidase and transferrin secretion [20]; and ionotropic P2X (2,3 and 7) ATP receptors that are present at different stages of the seminiferous epithelium cycle [21]. We have previously shown that Sertoli cells are also able to convert extracellular ATP into inosine by ectonucleotidase activity [22], and that cells of the seminiferous tubules differentially secrete extracellular nucleotides and their metabolites, suggesting a function for these molecules in paracrine signaling [23].

Several pieces of evidence have shown that purinergic receptors are linked to TNF-α signaling. Recent reports have described purinergic modulation of TNF-α signaling in neutrophils [24] and astrocytes [25]. Thus, in this article we examine the role of extracellular nucleotides in TNF-α signaling in Sertoli cells.

Although the effect of this cytokine in nitric oxide production has already been described in these cells, these results were obtained with interferon-γ and LPS treatment [26,27]. Since it has been reported that extracellular adenosine, inosine and ATP stimulate nitric oxide production in several cell types [28-31], we also examined the effects of TNF-α on the extracellular purines of cultured Sertoli cells and the role of these molecules in TNF-α stimulated nitric oxide production.

MATERIALS AND METHODS

Materials and animals

DMEM/F-12 medium was purchased from Gibco-BRL Life Technologies (Rockville, MD, USA). L-[2,3,4,5-^{3}H]arginine was purchased from Amersham Pharmacia Biotech (UK). TNF-α and all other drugs, reagents and enzymes were purchased from Sigma Chemicals (St. Louis, MO, USA). Pregnant Wistar rats were housed individually in plexiglass cages. Litters were restricted to eight pups each. The animals were maintained on a 12h light/dark cycle at a constant temperature of 23°C, with free access to commercial food and water. Male immature rats (18 days old) were killed by ether inhalation.

Isolation and culture of Sertoli cells

Sertoli cells were isolated as previously described [32], following the method of Tung and Fritz [33] with modifications. Testes of immature 18 day-old Wistar rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 34^{0}C, and centrifuged at 750 g for 5 min. The pellet was washed with soybean trypsin inhibitor, incubated with collagenase and hyaluronidase for 30 min at 34^{0}C, and then centrifuged at 40g for 10 min. The pellet was reincubated with hyaluronidase for 30 min at 34^{0}C and then centrifuged at 40g for 10 min. After counting, Sertoli cells were plated at a density of 3x10^{5} cells/cm^{2} in DMEM:F12 (1:1, low glucose) 1% FBS, supplemented with sodium bicarbonate, HEPES and gentamicin, and maintained in a humidified atmosphere of 5% CO_2 at 34^{0}C for 72 h. The medium was

changed every 24 h. Sertoli cell cultures were estimated to be 90-95% pure, as assessed by the alkaline phosphatase assay.

Extracellular purine nucleotides assay

To determine the production of purine nucleotides and their metabolites in the incubation medium we used the method described by Cunha *et al.*[34]. Cultured Sertoli cells were gently washed three times to remove medium and any dead or dying cells, and then incubated with HBSS (without phenol-red, 15 mM HEPES) for different periods of time in 5% CO_2 at 34^0C in the presence or absence of TNF-α and/or EHNA. After incubation, the medium was removed and centrifuged to eliminate debris. Samples were treated with TFA 7% to precipitate proteins, evaporated in a vacuum centrifuge (-61^0C) and resuspended in a reduced volume (10% of original). The purine content was determined in a reverse-phase HPLC system equipped with a C- 18 column (SupelcosilTM, Supelco®, 25 cm x 4.6 mm) and UV detector. The elution program was as follows: 10 min with 96% buffer A (KH_2P0_4 100 mM, pH 6.5) and 4% buffer B (buffer A plus methanol 30%), followed by a 5 min linear gradient up to 50% buffer B, hold for 10 min at a flow rate of 1.25 ml/min. UV absorption was measured at 245 nm. Internal standards were used for identification of purines.

To determine adenosine deaminase and ecto-5´-nucleotidase activities, control and TNF-α pre-treated cultured Sertoli cells were incubated with adenosine or 5´-AMP 25 μM in HBSS (without phenol-red, 15 mM HEPES) for 30 min. The degradation of adenosine or AMP was measured by HPLC as previously described.

Nitrite assay

To determine the amount of nitric oxide released by the Sertoli cells, the incubation medium was assayed for the stable end product of nitric oxide oxidation, nitrite (NO_2^-) [35], using Griess reagent. Cultured Sertoli cells were incubated for 24 h in MEM (phenol red free) 1% FBS with sodium bicarbonate, glucose, glutamine and gentamicin, in the presence or absence of TNF-α, EHNA or inosine. After incubation, supernatants were reacted with Griess reagent 1:1 for 15 min and the nitrite content was measured by absorbance at 540 nm. Concentration of nitrite in the samples was calculated using a standard curve prepared with $NaNO_2$ [27].

Nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was assayed in intact cultured Sertoli cells by measuring the conversion of L-[2,3,4,5-^{3}H]arginine into L-[^{3}H]citrulline (modified from Bulotta *et al.* [36]). Briefly, cultured Sertoli cells, pre-treated or not with TNF-α, EHNA or inosine, were incubated with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine in HBSS (without phenol-red, 15 mM HEPES) for 20 min and the cells were washed with ice-cold phosphate-buffered saline. Ethanol was added to the dishes and left to evaporate before a final addition of distilled water. Separation of L-[^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine was obtained by Dowex 50X8-400 chromatography as described by Salter *et al.*[37] and the L-[^{3}H]citrulline content was measured by liquid scintillation counting.

Protein quantification

All the results were standardized with respect to protein content, determined as described by Lowry *et al.* [38].

Statistical analysis

Results are expressed as means $\pm$ SEM of at least three dishes and are representative of three or more independent experiments. Differences between means were analyzed by ANOVA with the Student-Newman Keuls multiple comparisons test. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Purinergic modulation of TNF-α signaling has been described in neutrophils [24] and astrocytes [25], but the alteration of extracellular purinergic content by this cytokine has not been reported. We have previously shown that cultured Sertoli cells release ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine, uric acid and allantoin [23]. TNF-α treatment did not modify the concentration of any of these purines except inosine (data not shown) over any time period studied. This cytokine increased inosine concentration in 30 minutes (~50%), 3 h (~100%) and 6 h (~40%), with no effect at 24 hours, when the

concentration of this nucleoside was similar in control and cytokine-treated cells (Fig. 1A). After 3 hours of incubation, TNF-α modulation of extracellular inosine was dose-dependent (Fig. 1B).

To test if the inosine increase was due to an augmented rate of adenosine deamination, we incubated Sertoli cells with TNF-α in the presence of an adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). Figure 2 shows that EHNA prevented the TNF-α stimulated inosine increase in cultured Sertoli cells, suggesting that the increase in extracellular inosine is dependent on adenosine deaminase activity. However, no effect of TNF-α on this enzyme activity was observed (data not shown), suggesting that basal activity was sufficient to account for deamination of increased adenosine, leading to inosine accumulation in TNF-α treated cells.

The source of this increased adenosine utilized to accumulate inosine in TNF-α treated cells is not clear, and could result from several events, such as increased adenosine secretion and/or nucleotide degradation. Savic *et al.* [39], in cultured mesangial cells, and Kalsi *et al.* [40], in human endothelial cells, showed that TNF-α modulates ecto-5´-nucleotidase activity, suggesting that this cytokine could regulate the extracellular purinergic content in these cells. In Sertoli cells, no change in ecto-5´-nucleotidase activity was produced by TNF-α treatment (data not shown). Miki and Eddy [41] recently reported that TNF-R1 exhibits ATPase activity that is involved in the regulation of the aggregation of this receptor when activated by TNF-α. This ATPase activity could lead to an alteration in the intracellular purinergic content when TNF-α binds to TNF-R1, which, via equilibrative nucleotide/nucleoside transporters, could alter the extracellular purine content.

It is known that TNF-α in combination with interferon-γ and/or LPS increases nitric oxide production in cultured Sertoli cells [26,27], and recently it was demonstrated that TNF-α alone increases the expression of inducible nitric oxide synthase in these cells [42]. In this current report we show that TNF-α alone increases about 30% the nitrite accumulation in 24 hours (fig. 3a), with no effect at shorter times (data not shown); however nitric oxide synthase activity was already increased at 6 hours (~30% -fig. 3b). This lag time

between enzyme activity and nitrite accumulation could be explained by the low sensitivity of the nitrite assay. No effect of TNF-α on nitric oxide synthase activity was observed before 6 hours (data not shown).

Min *et al.* [29] reported that extracellular inosine increases LPS-stimulated nitric oxide production in macrophages. Since TNF-α increased extracellular inosine in cultured Sertoli cells, we studied the possible involvement of this nucleoside in cytokine-stimulated nitric oxide production in these cells. To this end, we investigated the effect of inosine on nitrite accumulation and nitric oxide synthase activity. Inosine increased both parameters in a very similar fashion to TNF-α (Fig. 4). To determine whether extracellular inosine has an intermediary role in TNF-α stimulated nitric oxide production, we utilized the adenosine deaminase inhibitor EHNA, which, as previously shown, inhibits cytokine-stimulated inosine accumulation. EHNA totally abrogates TNF-α stimulation of nitrite accumulation and the increase in nitric oxide synthase activity (Fig. 5). Extracellular inosine has been stated to act on A3 adenosine receptors in mast cells [43,44]; however, this receptor is not found in Sertoli cells [16,17]. In macrophages, extracellular inosine modulates LPS-stimulated nitric oxide production via an intracellular pathway [29], but this mechanism is not known to occur in Sertoli cells. Thus, more studies are necessary to determine the mechanism of action of this nucleoside in Sertoli cells.

Extracellular adenosine has been reported to mediate ethanol-induced nitric oxide production in hepatocytes [31] and modulates LPS-stimulated nitric oxide production in macrophages [29]. However, in Sertoli cells, extracellular adenosine, which was observed to be increased in EHNA and TNF-α+EHNA treated groups (Fig. 2), failed to induce nitric oxide production (Fig. 5). R-PIA, an A1 selective agonist, also failed to stimulate nitric oxide production (data not shown), excluding the participation of this receptor in the modulation of nitric oxide synthase in Sertoli cells.

Concluding, this is the first report to show that TNF-α increases extracellular inosine concentrations in cultured Sertoli cells. The results presented in this article suggests that this nucleoside can act as an intermediary in TNF-α induced nitric oxide production in these cells. More studies are necessary to better understand the physiological significance of this process.

LEGENDS

Figure 1. TNF-α induced increase of extracellular inosine content in cultured Sertoli cells. (A) Cultured Sertoli cells were incubated in the absence (white bars) or presence (black bars) of TNF-α 500 U/ml (25ng/ml) for the indicated periods of time (hours). (B) Cultured Sertoli cells were incubated for 3 h in the presence or absence of the indicated cytokine concentration. Inosine in the incubation medium was determined by HPLC. Results are presented as means ± S.E.M for at least three dishes and are representative of at least three independent experiments. * $p < 0.05$ as compared to respective control, ANOVA/Student-Newman Keuls post-hoc.

Figure 2. Extracellular inosine and adenosine content in cultured Sertoli cells. Cultured Sertoli cells were incubated in the absence or presence of TNF-α 500 U/ml (25ng/ml) and/or EHNA 10 μM for 6 h. Adenosine (white bars) and inosine (black bars) in the incubation medium were measured by HPLC. Results are presented as means ± S.E.M for at least three dishes and are representative of at least three independent experiments. * $p < 0.05$ respective to control; + $p < 0.05$ respective to EHNA group, ANOVA/Student-Newman Keuls post-hoc.

Figure 3. TNF-α induced increase of nitric oxide production in cultured Sertoli cells. (A) Cultured Sertoli cells were incubated in the absence or presence of TNF-α 500 U/ml (25ng/ml) for 24 h and nitrite accumulation in the medium was measured with Griess reagent. (B) Cultured Sertoli cells were incubated in the absence or presence of TNF-α 500 U/ml (25ng/ml) for 6 h and nitric oxide synthase activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Results are presented as means ± S.E.M for at least four (A) or three (B) dishes and are representative of at least three independent experiments. * $p < 0.05$ respective to control, ANOVA/Student-Newman Keuls post-hoc.

Figure 4. Exogenous inosine induced increase of nitric oxide production in cultured Sertoli cells. (A) Cultured Sertoli cells were incubated in the absence or presence of inosine 1mM for 24 h and nitrite accumulation in the medium was measured with Griess reagent. (B) Cultured Sertoli cells were incubated in the absence or presence of inosine 1mM for 6 h and nitric oxide synthase activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Results are presented as means ± S.E.M for at least four (A) or three (B) dishes and are representative of at least three independent experiments. * $p < 0.05$ respective to control, ANOVA/Student-Newman Keuls post-hoc.

Figure 5. Inhibition of adenosine deaminase abrogates TNF-α stimulated nitric oxide production in cultured Sertoli cells. (A) Cultured Sertoli cells were incubated in the absence or presence of TNF-α 500 U/ml (25ng/ml) and/or EHNA 10 μM for 24 h. Nitrite accumulation in the medium was then measured with Griess reagent. (B) Cultured Sertoli cells were incubated in the absence or presence of TNF-α 500 U/ml (25ng/ml) and/or EHNA 10 μM for 6 h and nitric oxide synthase activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Results are presented as means ± S.E.M for at least four (A) or three (B) dishes and are representative of at least three independent experiments. * $p < 0.05$ respective to control, ANOVA/Student-Newman Keuls post-hoc.

ACKNOWLEDGEMENTS

This work was supported by CAPES, CNPq, FAPERGS and PROPESQ/UFRGS.

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Figure 1 - Souza *et al.*

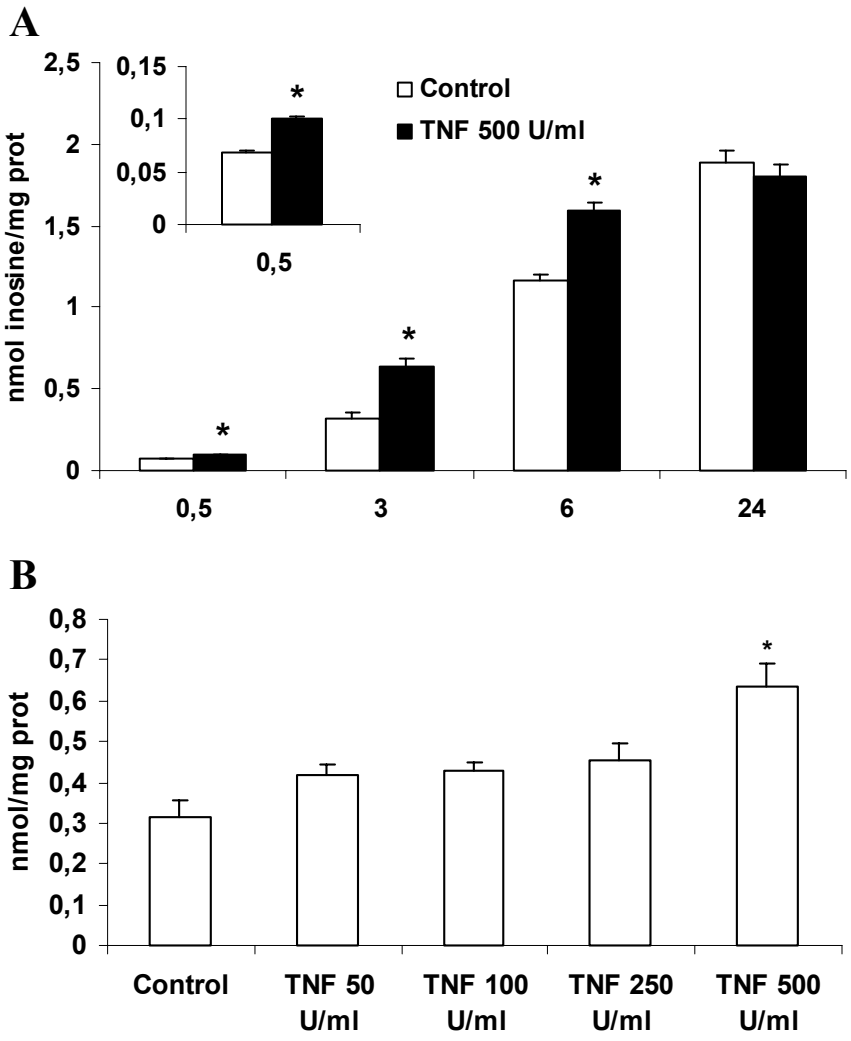


Figure 2 - Souza *et al.*

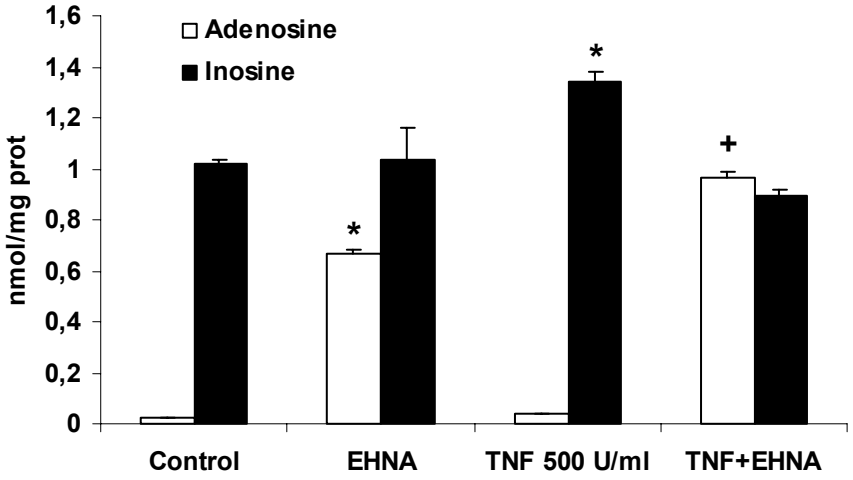


Figure 3 - Souza *et al.*

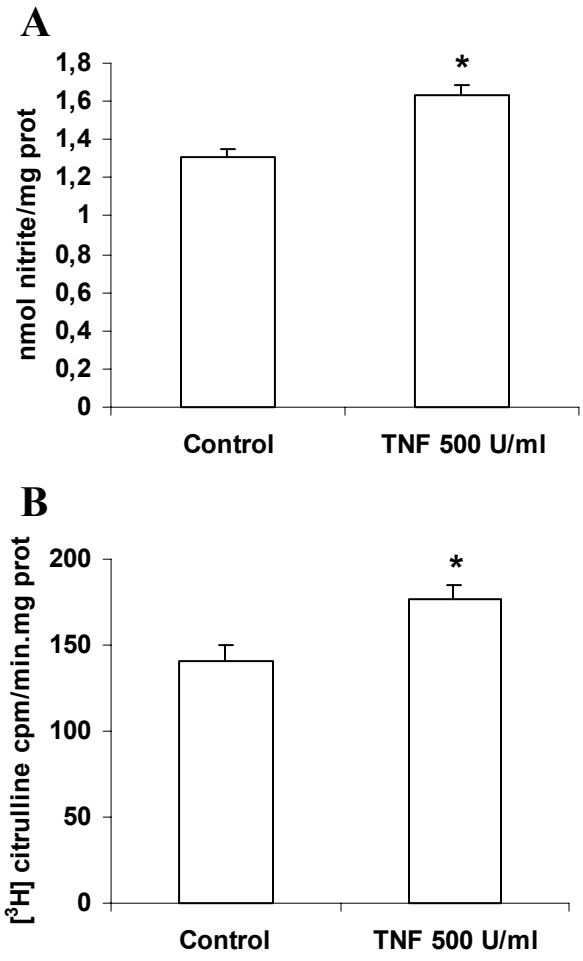


Figure 4 - Souza *et al.*

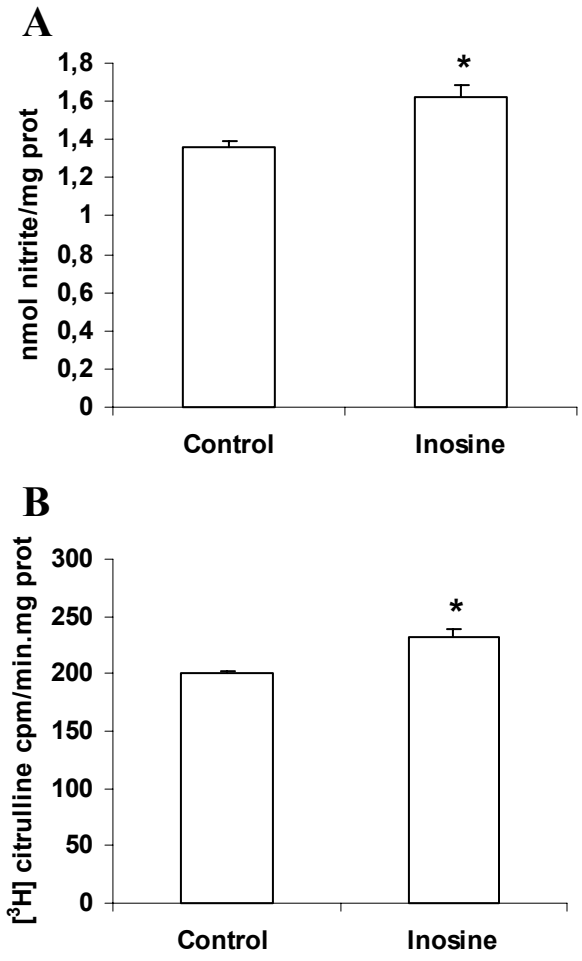
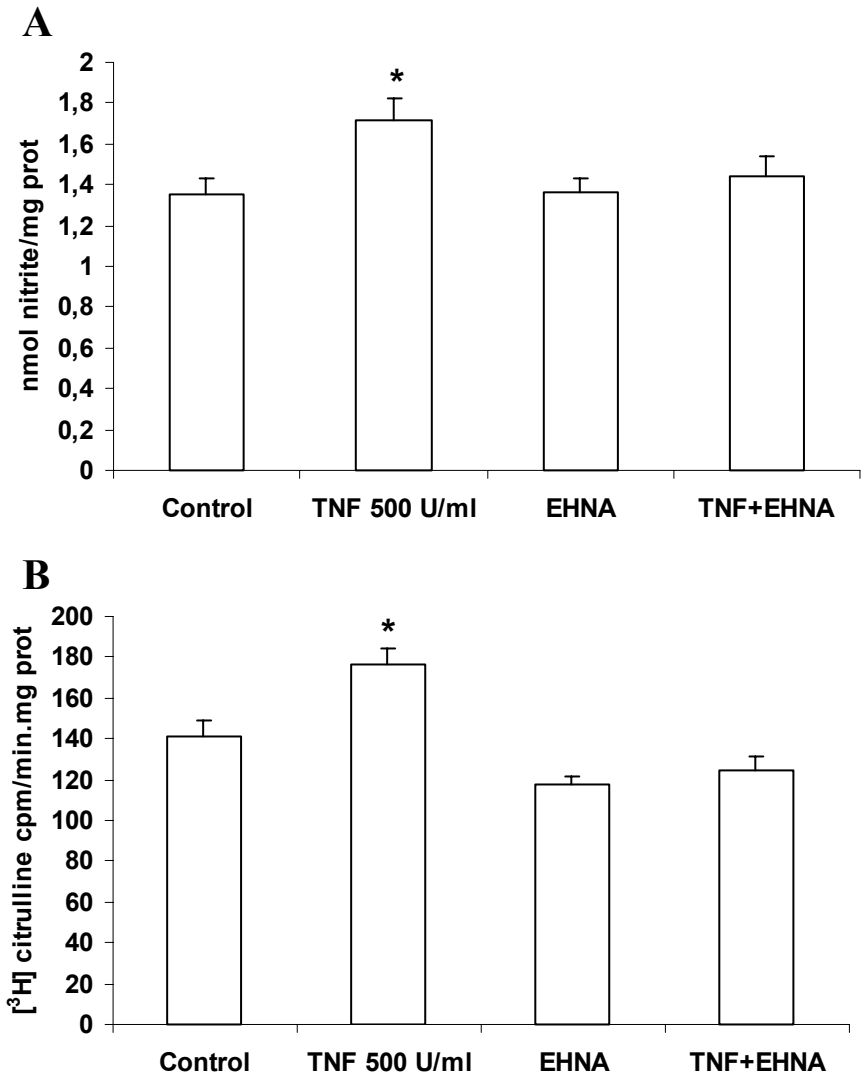


Figure 5 - Souza *et al.*



CAPÍTULO 3

Inosina extracelular modula a fosforilação das MAPKs ERK ½ e p38: possível papel na modulação da ERK ½ pelo TNF-α

Manuscrito a ser enviado para publicação na revista

Biochemical and Biophysical Research communications.

Extracellular inosine modulates ERK ½ and p38 phosphorylation in cultured Sertoli cells: possible participation in TNF-α modulation of ERK ½.

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Fax number: 55-51-3316.5535/5540

Abstract

Extracellular ATP and adenosine modulation of MAPKs is well described in different cells. However, it is not known effects of extracellular inosine on these kinases. Previous reports showed that hydrogen peroxide and TNF-α increases extracellular inosine concentration in cultured Sertoli cells. This nucleoside protects Sertoli cells against hydrogen peroxide induced damage and participates in TNF-α induced nitric oxide production on these cells. Considering that MAPKs are key mediators of cellular response to a great variety of stimulus, in this work it was investigated the effect of extracellular inosine in the phosphorylation of ERK ½ and p38 MAPKs in cultured Sertoli cells. Also it was investigated if this nucleoside could be involved in TNF-α modulation of ERK ½ phosphorylation. Extracellular inosine increase the phosphorylation of ERK ½ and p38. The selective A1 adenosine receptor agonist R-PIA also increase the phosphorylation of ERK ½ and p38 phosphorylation. Selective A1 adenosine receptors antagonists, CPT and DPCPX, inhibited extracellular inosine and R-PIA effects on ERK ½ and p38. These antagonists also inhibited TNF-α increase in the phosphorylation of ERK ½. Additionally, TNF-α rapidly augmented extracellular inosine concentration in cultured Sertoli cells. These results shown that extracellular inosine modulates ERK ½ and p38 in cultured Sertoli cells, possible trought A1 adenosine receptor activation. Besides, this nucleoside participates in TNF-α modulation of ERK ½.

Key words- extracellular inosine, TNF-α, MAPKs, ERK, p38 and Sertoli.

Introduction:

Extracellular purines are important regulators of many cellular functions. They activates distinct cell surface receptors, termed adenosine (or P1) and P2 receptors. P1 receptors are mainly responsive to adenosine and P2 receptors are mainly responsive to extracellular nucleotides. P1 or adenosine receptors are divided in four subtypes: A1, A2A, A2B and A3, which are all G protein-coupled receptors [1]. P2 receptors are divided in two distinct families: the P2X ligand-gated ionotropic channel family and the P2Y metabotropic G protein-coupled family [2,3]. Extracellular inosine, the degradative product of adenosine deamination, was initially considered an inactive metabolite of purines catabolism, however, recently many works has addressed the role of this nucleoside in the regulation of different processes. In mast cells, inosine stimulates degranulation via A_3 adenosine receptors, leading to increased vasopermeability in mice [4,5]. Also is reported the protective role of inosine in many cellular and animal models. In endotoxemia [6] and acute lung injury [7], inosine reduce the production of inflammatory citokines and increase the production of anti-inflammatory citokines by macrophages, improving the survival of experimental animals. Extracellular inosine also prevents glial cell death during glucose deprivation [8,9] and improves renal function in ischemia [10,11]. Besides, inosine protects RAW 264.7 macrophages of oxidant cell death by inhibiting poly (ADP-ribose) polimerase activity [12].

Sertoli cells are the somatic cells of seminiferous tubules, responsable for energetic supply, mechanical support and hormonal control of spermatogenesis. These cells express distinct purinoreceptors: A1 adenosine receptor, that were related with the inhibition of FSH increased cAMP concentration [13,14,15]; P2Y2 ATP receptor, which regulates phosphatidyl-inositol turnover and calcium mobilization, inhibiting cAMP accumulation [16] and increasing γ-glutamyl-transpeptidase and transferrin secretion [17]; and ionotropic P2X(2,3 and 7) ATP receptors that are differentially present at distinct stages of seminiferous epithelium cycle [18]. In addition, previous works reported that Sertoli cells shown different ectonucleotidases activities responsible for extracellular purine metabolism [19] and that these cells secretes extracellular nucleotides and their metabolites, including inosine [20], which was demonstred to be involved in TNF-α modulation of nitric oxide production [21] and protection against H_2O_2 induced lipoperoxidation and death [22].

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of cell cycle progression, proliferation, differentiation and death. The MAPK family consists of three main

subgroups: the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases (SAPKs) p38 and the SAPK c-jun N terminal kinases (JNKs). The modulation of MAPKs by extracellular adenosine acting in any of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) trough different mechanisms and in distinct cells is well described [revised in 23]. Besides, extracellular ATP is also described to modulate MAPKs trough P2 receptors [24,25,26,27]. However, the action of extracellular inosine on these kinases is not reported. In Sertoli cells, ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation [28] and transforming growth factor β 3 regulates distinct Sertoli functions via MAPKs. [29]. Furthermore, TNF-α activates ERK ½, p38 and JNK MAPKs, regulating interleukine-6 production, via p38, and intercellular adhesion molecule-1 expression, via JNK [30,31].

In this work, it was investigated if extracellular inosine modulates ERK ½ and p38 MAPKs in cultured Sertoli cells, studying the possible receptor involved. Additionally, was investigated the role of extracellular inosine in the TNF-α modulation of ERK ½.

Material and Methods

Material and animals

DMEM/F-12 medium was purchased from Gibco-BRL Life Technologies (Rockville, MD, USA). Anti phospho-ERK ½ and anti phospho-p38 antibodies were purchased from Santa Cruz (USA). TNF-α, R-PIA, CPT, DPCPX, Inosine and all other drugs, reagents and enzymes were purchased from Sigma Chemicals (St. Louis, MO, USA). Pregnant Wistar rats were housed individually in plexiglass cages. Litters were restricted to eight pups each. The animals were maintained on a 12h light/dark cycle at a constant temperature of 23°C, with free access to commercial food and water. Male immature rats (18 days old) were killed by ether inhalation.

Isolation and culture of Sertoli cells

Sertoli cells were isolated as previously described [32], following the method of Tung and Fritz [33] with modifications. Testes of immature 18 day-old Wistar rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 34^0C, and centrifuged at 750 g for 5 min. The pellet was washed with soybean trypsin inhibitor, centrifuged and incubated with collagenase and hyaluronidase for 30 min at 34^0C, then was centrifuged at 40g for 10 min. After this, the pellet was

reincubated with hyaluronidase for 30 min at 34^0C and then centrifuged at 40g for 10 min. After counting, Sertoli cells were plated in a density of $3x10^5$ cells/cm^2 in DMEM:F12 (1:1, low glucose) 1% FBS, supplemented with sodium bicarbonate, HEPES and gentamicin, and maintained in a humidified atmosphere of 5% CO_2 at 34^0C for 72 h. The medium were changed every 24 h. Sertoli cell cultures were estimated to be 90-95% pure, as assessed by alkaline phosphatase assay.

Western blots: ERK ½ and p38 phosphorylation

Cultured Sertoli cells were harvested with a rubber policeman, centrifuged and lysed in 1% Triton/PBS. Equal amounts of protein (~50μg) were resolved by SDS-PAGE 12%, transferred to nitrocellulose and reacted with specific antibodies for phosphorylated forms of ERK ½ and p38. Peroxidase tagged secondary antibodies anti immunoglobulin was used and the detection was performed using the enhanced chemiluminescence system (ECL). Immunoblottings were quantified by densitometric scanning of films and all results were representative of two or more independent experiments.

Extracellular purine nucleotides assay

To investigate purine nucleotides and their metabolites in incubation medium we used the method described by Cunha *et al.*[34]. Cultured Sertoli cells were gentle washed three times to remove remnants of medium and eventual dead or dying cells, and then incubated with HBSS (without phenol-red, 15 mM HEPES) in the presence or absence of TNF-α in 5% CO_2 at 34^0C. After incubation the medium was removed and centrifuged to eliminate debris. Samples were treated with TFA 7% to precipitate proteins, evaporated in vacuum centrifuge (-61^0C) and resuspended in a reduced volume (10% of original volume). The sample purine contents were determined by a reverse-phase HPLC system equipped with a C- 18 column (SupelcosilTM, Supelco®, 25 cm x 4,6 mm) and UV detector. The elution program consists in: 10 min with 96% buffer A (KH_2P0_4 100 mM, pH 6.5) and 4% buffer B (buffer A plus methanol 30%), followed by a 5 min linear gradient up to 50% of buffer B, and hold for 10 min, at a flow rate of 1,25 ml/min (UV absorption of 245 nm). To identify the substances detected, each sample was loaded with standard solutions.

Protein quantification

All the results were standardized by the protein content, determined as described by Lowry *et al.* [35].

Results

In previous works, it was described that cultured Sertoli cells secretes inosine to extracellular medium, leading to the accumulation of this purine [20]. It was also described that TNF-α and hydrogen peroxide increases the extracellular concentration of inosine [21,22]. MAPKs cascades are involved in the response to different stimulus, like growth factors, cytokines and oxidative stress [23,36]. To investigate if extracellular inosine could modulates the activation of ERK ½ , the phosphorylation of this MAPK in cultured Sertoli cells treated with inosine were studied. Extracellular inosine increased ERK ½ phosphorylation with maximal effect at 5 minutes, decreasing this effect in longer times (Figure 1A). The increase in phosphorylation of ERK ½ is already observed with concentration of 0,1 mM of inosine and is maximal with 1 mM (data not shown).

Extracellular inosine is described to activate A3 adenosine receptor in mast cells [4,5], however, only A1 adenosine receptor is described in Sertoli cells [13,14]. To study if A1 adenosine receptor could be involved in ERK ½ activation, Sertoli cells were incubated with inosine or a selective A1 adenosine receptor agonist, R-PIA ((R)-N6-(2-phenylisopropyl)-adenosine), in the presence or ausence of specific A1 adenosine receptor antagonists, CPT (cyclopentil-teophyline) and DPCPX (8-cyclopentil-1,3-dipropylxanthine). The A1 antagonists inhibited both extracellular inosine and R-PIA increased phosphorylation of ERK ½ (Figure 2).

TNF-α is described to activates ERK ½ in cultured Sertoli cells [30] and, in a previous work, it was demonstred that this cytokine increases extracellular inosine concentration in these cells [21]. Figure 3 showns that TNF-α rapidly increases extracellular inosine at 5 minutes of incubation. To verify the participation of this nucleoside in cytokine enhanced phosphorylation of ERK ½, Sertoli cells were incubated with TNF-α in the presence of selective A1 antagonists. Figure 4 showns that CPT and DPCPX completely abbrogates the effect of cytokine.

The effect of extracellular inosine in p38 phosphorylation was also investigated. This nucleoside increased p38 phosphorylation in time dependent way, similar to ERK ½ modulation, with maximal effect at 5 minutes (Figure 5A). The selective antagonist CPT inhibited extracellular inosine effect in p38 phosphorylation (Figure 5B)

Discussion

In this work, it was reported the modulation of ERK ½ and p38 phosphorylation in cultured Sertoli cells by extracellular inosine. Adenosine modulation of MAPKs via all four adenosine receptors is described. These effects has been studied in cells that usually express adenosine receptors and in cells stably transfected with adenosine receptors [revised in 23]. It is described that extracellular inosine activates A3 adenosine receptor in mast cells [4,5], however, only A1 adenosine receptor has been described on Sertoli cells [13,14]. The inhibition of extracellular inosine action by selective A1 antagonists CPT and DPCPX, and the modulation of ERK ½ and p38 phosphorylation by A1 selective agonist R-PIA suggests that extracellular inosine could be activating A1 adenosine receptor in Sertoli cells. Taking in account that in mast cells the binding of extracellular inosine to this receptor is negligible [15], this could suggests that Sertoli cells express a different subtype of A1 adenosine receptor when compared to mast cells.

In a previous work, the increase of extracellular inosine by hydrogen peroxide was described. It was also demonstred that this nucleoside protects Sertoli cells against oxidative stress induced damage [22]. The activation of ERK ½ is described to be involved in the protection against oxidative induced cell death in cAMP potentiation of noradrenaline neuroprotection on dopaminergic neurons [37], estrogen neuprotection [38] and calcitonin gene-related peptide protection in cultured smooth muscle cells [39]. In Sertoli cells, the inosine stimulated phosphorylation of ERK ½, described in this work, could be involved in the protection against hydrogen peroxide previously cited. Furthermore, it is described that oxidative stress modulates MAPKs [36], suggesting that extracellular inosine could participates in hydrogen peroxide modulation of Sertoli cell functions.

In testis, TNF-α is produced, in physiological condition, principally by round spermatids [40], exerting a regulatory function on spermatogenesis. However, in inflammatory responses, interstitial macrophages can account for considerable production of this cytokine [41,42]. Sertoli cells expresses both TNF-α receptors [43,44] and this cytokine modulates diverse functions of these cells related to the maintenance of spermatogenesis, including lactate production [45] and interleukin-6 [46], inhibin [47], transferrin [48] and insulin–like growth factor binding protein 3 expression [49]. In addition, in Sertoli cells TNF-α modulates distinct FSH actions [50], Fas-ligand system [51] and tight junction dynamics [52].

The modulation of ERK ½ phosphorylation by TNF-α in Sertoli cells is described [30]. In a previous work, it was reported that TNF-α increases extracellular inosine content in cultured Sertoli cells. It was also showed that this increment in the extracellular nucleoside concentration are related to increased nitric oxide production [21]. In this work, we showed that TNF-α stimulated increase in extracellular inosine content is very rapidly, supporting that this nucleoside are acting as a mediator in ERK ½ modulation. This is supported by the results with selective A1 adenosine receptor antagonists CPT and DPCPX, which inhibits citokyne stimulated ERK1/2 phosphorylation. Miki and Eddy [53] recently reported that TNF-R1 exhibits ATPase activity that are involved in the regulation of the aggregation of this receptor when activated by TNF-α. This ATPase activity could leads to alteration in the intracellular purines content when TNF-α binds to TNF-R1, which, via equilibrative nucleotide/nucleoside transporters, could alters extracellular purines content. No alteration of extracellular adenosine concentration was observed in TNF-α treated Sertoli cells, discarding a possible participation of this nucleoside.

Concluding, this is the first report to shown that extracellular inosine increases ERK ½ and p38 phosphorylation in Sertoli cells, possible trought A1 adenosine receptor activation. Besides, it is showed that TNF-α rapidly increases extracellular inosine concentration and that A1 antagonists blocks TNF-α induced phosphorylation of ERK ½, suggesting a participation of extracellular inosine in cytokine signalling.

ACKNOWLEDGEMENTS

This work was supported by CAPES, CNPq, FAPERGS and PROPESQ/UFRGS.

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Figure Legends

Figure 1: Time dependence of extracellular inosine modulation of ERK ½ phosphorylation. Cultured Sertoli cells were incubated for the indicated time periods in the presence of extracellular inosine 1mM. Cell lysates (50 μg) were resolved by SDS/PAGE and transferred to nytrocellulose before being probed with specific anti phospho-ERK ½. Representative Western blot of three independent experiments are shown.

Figure 2: Effect of selective A1 adenosine receptors antagonists on extracellular inosine and A1 adenosine receptor agonist modulation of ERK ½ phosphorylation. Cultured Sertoli cells were incubated for 5 minutes in the presence or not of CPT 100μM (A) or DPCPX 100μM (B) (A1 antagonists) before incubation for 5 minutes in the presence or not of extracellular inosine 1mM or R-PIA 100μM (A1 agonist). Cell lysates (50 μg) were resolved by SDS/PAGE and transferred to nytrocellulose before being probed with specific anti phospho-ERK ½. Representative Western blot of three independent experiments are shown.

Figure 3: TNF-α modulation of extracelular inosine concentration. Cultured Sertoli cells were incubated for 5 minutes in the presence of TNF-α 500 U/ml (25ng/ml). Extracellular inosine in the incubation medium were measured by HPLC. Results are presented as mean $\pm$ S.E.M for at least or three dishes and are representative of three independent experiments. * $p < 0,05$ respectively to control, ANOVA/Student-Newman Keuls post-hoc.

Figure 4: Effect of selective A1 adenosine receptors antagonists on TNF-α modulation of ERK ½ phosphorylation. Cultured Sertoli cells were incubated for 5 minutes in the presence or not of CPT 100μM or DPCPX 100μM (A1 antagonists) before incubation for 15 minutes in the presence of TNF-α 500 U/ml (25ng/ml). Cell lysates (50 μg) were resolved by SDS/PAGE and transferred to nytrocellulose before being probed with specific anti phospho-ERK ½. Representative Western blot of three independent experiments are shown.

Figure 5: Extracellular inosine modulation of p38 phosphorylation and effect of selective A1 adenosine receptors antagonist. (A) Cultured Sertoli cells were incubated for the indicated time periods in the presence of extracellular inosine 1 mM. (B) Cultured Sertoli cells were incubated for 5 minutes in the presence or not of CPT 100μM (A1 antagonist) before incubation for 5 minutes in the presence of extracellular inosine 1mM. Cell lysates (50 μg) were resolved by SDS/PAGE and transferred to nytrocellulose before being probed with specific anti phospho-p38. Representative Western blot of three independent experiments are shown.

Figure 1

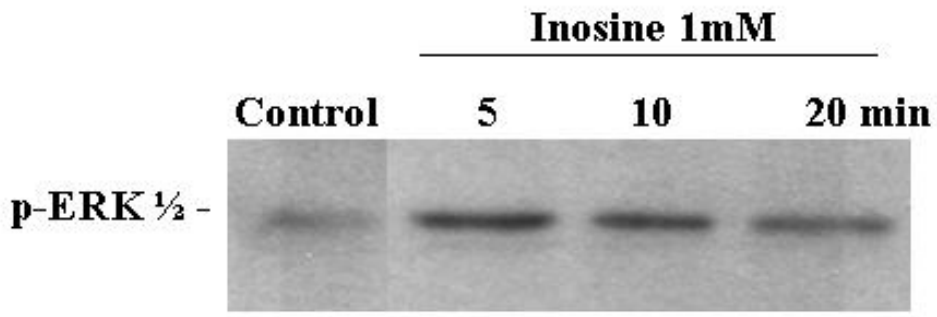


Figure 2

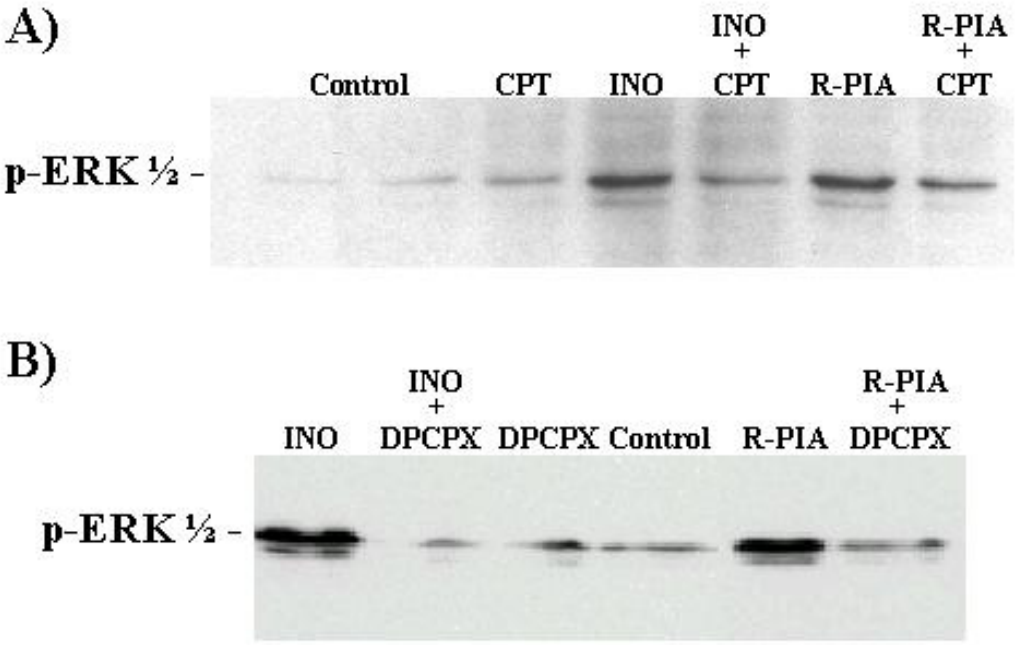

A)
Control
CPT
INO
INO + CPT
R-PIA
R-PIA + CPT
p-ERK ½ -
B)
INO
INO + DPCPX
DPCPX
Control
R-PIA
R-PIA + DPCPX
p-ERK ½ -

Figure 3

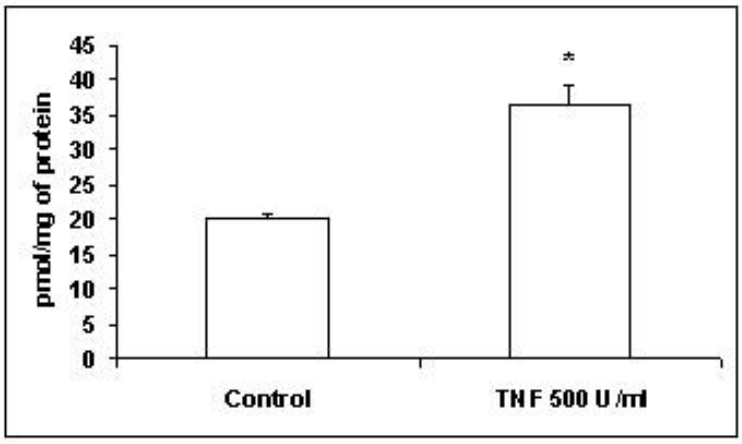


Figure 4

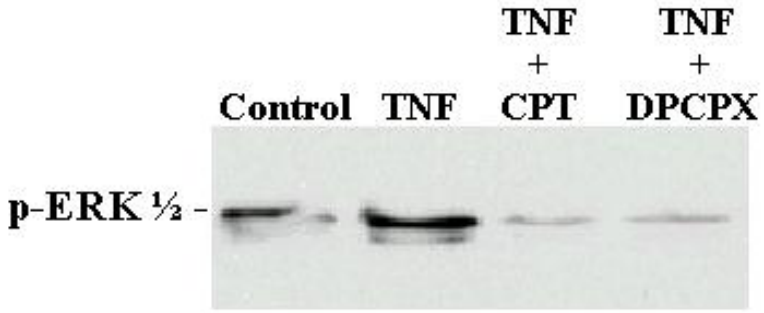
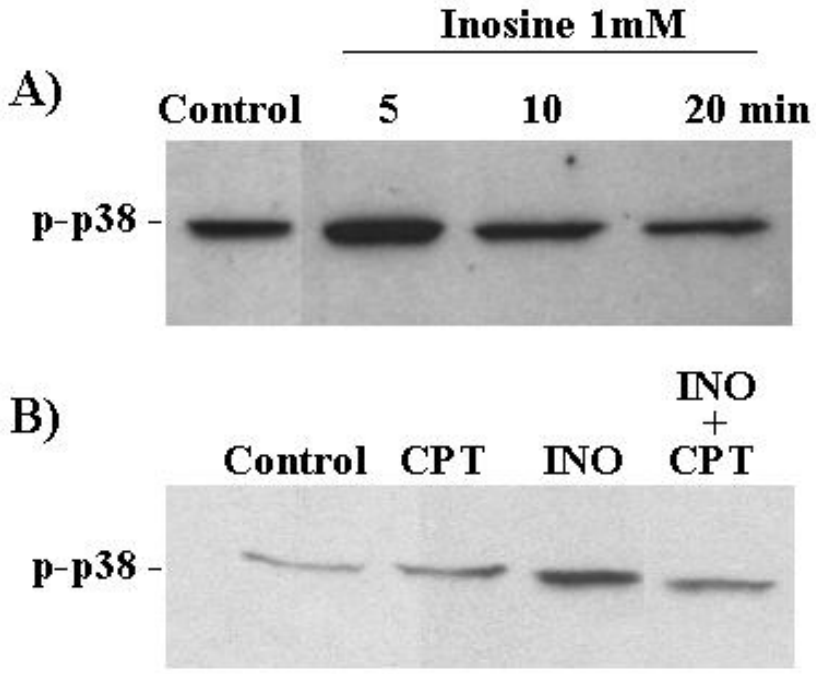


Figure 5


Inosine 1mM
A)
Control
5
10
20 min
p-p38
B)
Control
CPT
INO
INO + CPT
p-p38

CAPÍTULO 4

DISCUSSÃO E CONCLUSÃO

4.1 - Discussão e Conclusão

Nesta dissertação é descrita a ação do TNF-α sobre o metabolismo das purinas extracelulares em células de Sertóli em cultura, onde esta citocina leva a um rápido aumento na concentração de inosina extracelular que se prolonga até seis horas. Além disso, é mostrado um papel intermediário da inosina extracelular na modulação da produção de óxido nítrico e no aumento da fosforilação da ERK ½ pelo TNF-α.

Como descrito no Capítulo 1, o TNF-α altera apenas a concentração extracelular da inosina, não alterando a concentração dos demais nucleotídeos e de seus metabólitos em condições normais. Entretanto, na presença de um inibidor da Adenosina Deaminase (ADA), enzima responsável pela deaminação da adenosina à inosina, ocorre um aumento da concentração de adenosina, como descrito anteriormente (Gelain *et al* 2003). Quando as células de Sertóli são incubadas com TNF-α na presença do inibidor da ADA, o acúmulo de adenosina é aumentado, enquanto que a concentração de inosina não se altera, mostrando que o acúmulo de inosina provocado pelo TNF-α é dependente da degradação da adenosina pela ADA. Entretanto, novos estudos são necessários a fim de se identificar a origem da adenosina degradada a inosina, isto é, se ela é secretada diretamente ou se é proveniente da degradação extracelular de outros nucleotídeos. Embora o aumento da concentração extracelular de inosina seja dependente da degradação de adenosina, o TNF-α não altera a atividade da ADA. Também não é encontrada alteração da atividade da ecto-5´-nucleotidase nas células de Sertóli tratadas com TNF-α, o que é descrito em células mesangiais (Savic *et al.* 2000) e em células endoteliais humanas (Kalsi *et al.* 2002).

É descrito que a inosina aumenta a produção de NO estimulada por LPS em macrófagos (Min *et al.* 2000), bem como, é descrito o aumento da expressão da NOS

induzível em células de Sertóli pela ação do TNF-α (Lee e Cheng 2003). Além disso, o TNF-α em combinação com interferon-γ e LPS aumenta a produção de NO nestas células (Stéphan *et al.* 1995, Bauché *et al.* 1998). No capítulo 1, é mostrado que a inosina extracelular e o TNF-α aumentam a produção de NO em células de Sertóli. Além disso, é mostrado que o inibidor de ADA, o qual previne a acumulação de inosina extracelular estimulada pela citocina, impede o aumento da produção de NO nas células de Sertóli tratadas com TNF-α. Logo, é sugerido um papel intermediário para a inosina extracelular na modulação da produção de NO pelo TNF-α nestas células.

No capítulo 2 desta dissertação, é mostrado o aumento da fosforilação da ERK ½ e da p38 pela inosina extracelular. Diversos trabalhos já haviam descrito a modulação das MAPKs por adenosina e ATP (Sellers *et al.* 2001, Shigemoto-Mogami *et al.* 2001, Bradford e Soltoff 2002, Gendron *et al.* 2003, Schulte e Fredholm 2003), no entanto, não era conhecida a ação da inosina nestas proteínas. Em mastócitos, a inosina ativa receptores de adenosina A3, estimulando a degranulação destas células (Jin *et at.*1997, Tilley *et al.* 2000). Embora a modulação das MAPKs pela adenosina seja descrita através da ativação dos quatro tipos de receptores para este nucleosídeo (revisado em Schulte e Fredholm 2003), as células de Sertóli expressam apenas o receptor para adenosina A1 (Monaco e Conti 1986, Rivkees 1994), o qual é descrito como não responsivo à inosina em mastócitos (Jin *et at.*1997). Entretanto, os antagonistas específicos para este receptor inibiram a ação da inosina na fosforilação da ERK ½ e da p38. Além disso, um agonista específico para o receptor de adenosina A1 também aumentou a fosforilação da ERK ½. Estes dados indicam que nas células de Sertóli o receptor de adenosina A1 é responsivo à inosina.

Previamente, havia sido descrito que o peróxido de hidrogênio aumenta a concentração extracelular da inosina em células de Sertóli (Gelain *et al.* 2004) e nesta dissertação é mostrado que o TNF-α também aumenta a concentração deste nucleosídeo nestas células. A modulação das MAPKs por estresse oxidativo é descrita em alguns tipos celulares (Lander 1997), e em células de Sertóli o TNF-α modula a fosforilação destas proteínas (De Cesaris *et al.* 1998). Assim, a inosina extracelular pode agir como uma mediadora da ação de diferentes estímulos na modulação das MAPKs. No capítulo 2, é mostrado que os antagonistas específicos para o receptor de adenosina A1 anulam o efeito do TNF-α na fosforilação da ERK ½, além de ser mostrado que esta citocina rapidamente aumenta a concentração extracelular de inosina. Estes resultados reforçam a idéia de que este nucleosídeo esteja agindo como um intermediário na sinalização do TNF-α.

Embora a inosina extracelular esteja envolvida na regulação da produção de NO e na fosforilação da ERK ½, diferentes mecanismos parecem estar envolvidos. A modulação da produção de NO pela inosina não envolve o receptor para adenosina A1, uma vez que o agonista específico para este receptor não exerce papel semelhante a inosina neste parâmetro, como citado no capítulo 1. A modulação da produção de NO em macrófagos pela inosina extracelular é dependente do transporte deste nucleosídeo para o interior da célula (Min *et al.* 2000), no entanto, a presença deste mecanismo nas células de Sertóli precisa ser investigado. Além disso, o inibidor da ADA, que aumenta a concentração extracelular de adenosina, não possui efeito na produção de NO. Por outro lado, o agonista do receptor para adenosina A1 aumenta a fosforilação da ERK ½, além de os antagonistas deste receptor inibirem a ação da inosina na fosforilação da ERK ½ e da p38, o que evidencia a participação do receptor para adenosina A1 nestes efeitos.

Como sumarizado na figura 2, o TNF-α aumenta a concentração extracelular de inosina em células de Sertóli em cultura. Além disso, este nucleosídeo atua como um intermediário na modulação da produção de NO e da fosforilação da ERK ½ nestas células.

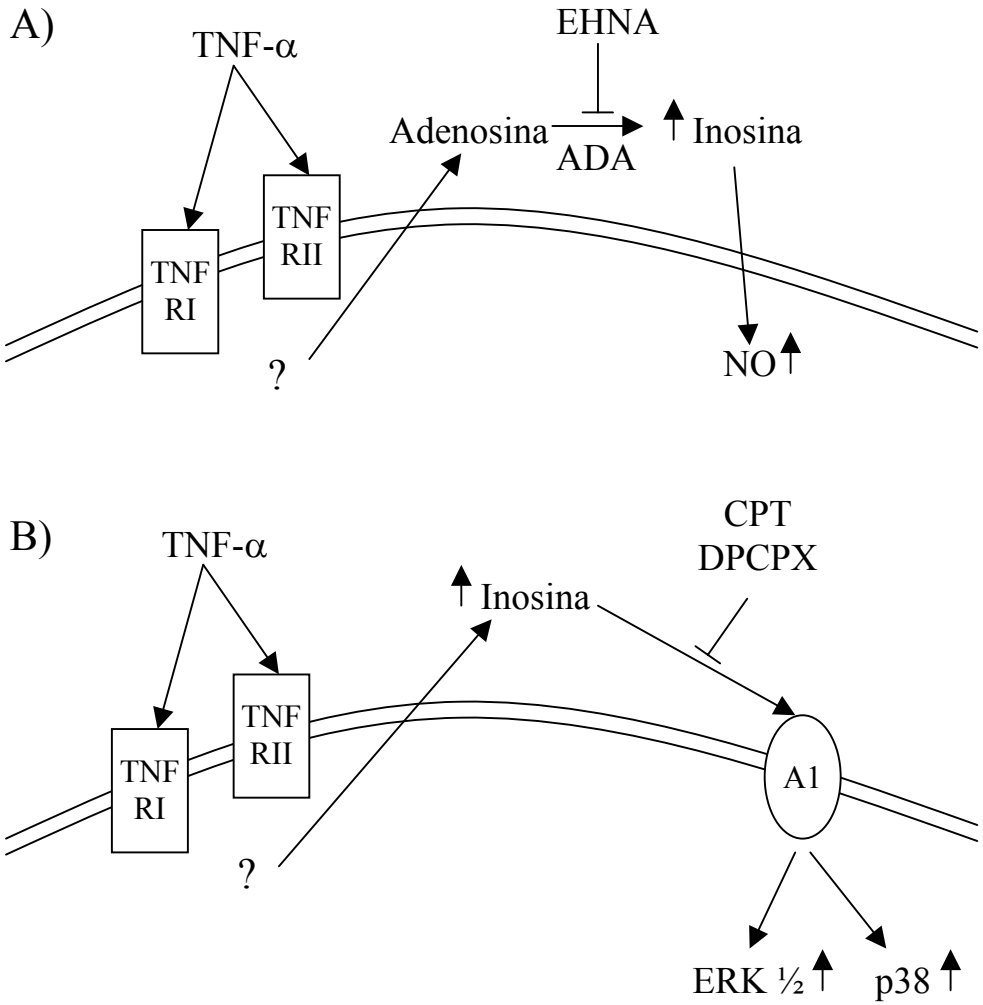


Figure 2: Representação esquemática dos resultados.
(A) Capítulo 2. (B) Capítulo 3.

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[3] O.R. Mettam, L.B. Adams, in: E.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 1999, pp. 218-304.
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