Free Radical Research, Volume 38 Number 1 (January 2004), pp. 37–47

Taylor & Francis
healthsciences

Extracellular Inosine is Modulated by H_2O_2 and Protects Sertoli Cells against Lipoperoxidation and Cellular Injury

DANIEL PENS GELAIN[a], LUIZ FERNANDO DE SOUZA[a], GISELE RONCHETI RIBEIRO[a], MARCELO ZIM[a], FERNANDA RAFAELA JARDIM[a], JOSÉ CLÁUDIO FONSECA MOREIRA[b] and ELENA AIDA BERNARD[a,*]

[a]*Laboratório de Transdução de Sinal em Células Testiculares, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil;* [b]*Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*

Accepted by Professor T. Finkel

(Received 5 May 2003; In revised form 30 July 2003)

Extracellular purines are involved in the regulation of a wide range of physiological processes, including cytoprotection, ischemic preconditioning, and cell death. These actions are usually mediated via triggering of membrane purinergic receptors, which may activate antioxidant enzymes, conferring cytoprotection. Recently, it was demonstrated that the oxidative stress induced by cisplatin up-regulated A_1 receptor expression in rat testes, suggesting an involvement of purinergic signaling in the response of testicular cells to oxidant injury. In this article, we report the effect of hydrogen peroxide on purinergic agonist release by cultured Sertoli cells. Extracellular inosine levels are strongly increased in the presence of H_2O_2, suggesting an involvement of this nucleoside on Sertoli cells response to oxidant treatment. Inosine was observed to decrease H_2O_2-induced lipoperoxidaton and cellular injury, and it also preserved cellular ATP content during H_2O_2 exposure. These effects were abolished in the presence of nucleoside uptake inhibitors, indicating that nucleoside internalisation is essential for its action in preventing cell damage.

Keywords: Hydrogen peroxide; Inosine; Purinoceptors; Sertoli cells; Lipoperoxidation; Oxidative stress

INTRODUCTION

Purine nucleosides and nucleotides are widely distributed molecules that exhibit a diverse range of effects in many cellular types, acting as important extracellular signals in addition to their more established roles in cellular metabolism.[1] They mediate their effects via activation of distinct cell surface receptors, termed adenosine (or P1) and P2 purinergic receptors.[2,3] While P1 receptors are activated by adenosine, P2 receptors are triggered mainly by ATP. More recently, it was demonstrated that the product of metabolic deamination of adenosine, inosine, is able to trigger A_3 adenosine receptors.[4]

Several pieces of evidence have shown that purinergic receptors are involved in the cellular response to oxidative stress. The activation of A_3 receptors in rat basophilic leukemia cells (RBL-2H3) leads to a 2–3-fold increase in activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and also increases the activity of glutathione reductase.[5] It is well known that adenosine has cardioprotective effects in ischemic heart disease.[6–9] Only recently it was demonstrated that this protective effect is due to an up-regulation in the expression of manganese-SOD[10] and a reduction in the reperfusion-derived production of reactive oxygen species (ROS), mediated by the opening of K_{ATP} channels via A_1 receptor activation.[11] Adenosine-derived ischemic preconditioning in the central nervous system (NS) has also been suggested to be correlated to cytoprotection against ROS, since free radicals can up-regulate A_1 receptor expression via NF-kB activation.[12] Triggering of A_1 and A_{2A} receptors attenuates H_2O_2 injury in kidney proximal tubular cells,[13] and adenosine can prevent

*Corresponding author. Address: Dep. Bioquímica (ICBS-UFRGS), Rua Ramiro Barcelos, 2600 anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55-51-3316-5548. Fax: +55-51-3316-5535. E-mail: elenbern@vortex.ufrgs.br

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760310001629056

the increase in the permeability of oxidant-injured endothelial cells via A_1 receptor activation.[14]

Sertoli cells are the main cellular component of the seminiferous tubules. They form the blood-testis barrier that isolates germinative cells within a specific microenvironment, where meiosis and spermiogenesis occur, supporting the germ cells, both metabolically and mechanically, during spermatogenesis.[15] These cells are also targets for follicle-stimulating hormone (FSH), testosterone and retinol, which regulates some of their biological properties related to spermatogenesis.[15,16] It is well known that Sertoli cells express A_1 purinoceptors related to the inhibition of the FSH-stimulated accumulation of cAMP,[17] while A_1 and A_3 receptors have been detected in germinative cells.[18] Recently, our group demonstrated that Sertoli cells release ATP to the extracellular environment, while germinative cells secrete adenosine;[19] both cell types can secrete significant amounts of inosine to the extracellular environment. These data indicate that purinergic signalling may be involved in biochemical communication between these cells. Thus extracellular purines have a very important role in the regulation of the biochemical properties of germinative and Sertoli cells.

Sertoli cells play a major role in iron metabolism in seminiferous tubules. They express and secrete significant amounts of transferrin. These cells are also active phagocytes, engulfing and lysing residual bodies which detach from spermatids,[20] and it has long been reported that phagocytes contain enzymes (such as NADPH oxidases and myeloperoxidases) which produce ROS during the process of phagocytosis.[21] Oxidative stress affects spermatozoa in complex ways; ROS has been reported to decrease sperm mobility, leading to male infertility.[22–24] Thus protection against oxidative stress is a prerequisite for the production of functional sperm. In fact, protection from ROS in testes is mediated by both antioxidant enzymes (SOD, CAT, GPx) and antioxidants such as ascorbate, alpha-tocopherol, glutathione and carotenes.[25] However, little is known about the mechanisms involved in the regulation of the antioxidant defence in physiological and pathophysiological processes producing ROS.

Recently, it was demonstrated that oxidative stress induced by administration of the chemotherapeutic agent cisplatin in rat testes increases the expression of adenosine A_1 receptors,[26] suggesting a link between purinergic signalling and the response of testicular cells to oxidative stress. The present article reports the changes in extracellular purines released by Sertoli cells during the oxidative stress induced by hydrogen peroxide (H_2O_2) and the effects on cellular viability and lipoperoxidation.

MATERIALS AND METHODS

Materials and Animals

All drugs, kits and enzymes were purchased from Sigma Chemical Co. (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 12-h 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,[27] following the method of Tung and Fritz.[28] Testes of immature rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 34°C, and centrifuged at 750 *g* for 5 min. The pellet was mixed with soybean trypsin inhibitor, then centrifuged and incubated with collagenase and hyaluronidase for 30 min at 34°C. After incubation, this fraction was centrifuged (10 min at 40 *g*). The pellet was taken to isolate Sertoli cells and the supernatant was discarded. After counting, Sertoli cells were plated in 6 × dishes multiwell plates (3×10^5 cells/cm^2) in DMEM/F12 (1:1, low glucose) 1% FBS, supplemented with sodium bicarbonate, HEPES and gentamicin, and maintained in a humidified 5% CO_2 atmosphere at 34°C for 24 h to attach. The medium was then changed to serum-free DMEM/F12 and cells were taken for assay after 48 h of culture. Sertoli cell cultures were estimated to be 90–95% pure, as assessed by the alkaline phosphatase assay.

Assays

To evaluate the effects of oxidative stress on the release of purinergic agonists, Sertoli cells were gently washed three times to eliminate debris and dead/dying cells and incubated for various times in 5% CO_2 at 34°C with phenol red-free HBSS supplemented with HEPES 15 mM in the presence or absence of H_2O_2 (25–200 μM). The incubation medium was used for analysis of extracellular purines. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) 10 μM was used to inhibit adenosine deaminase (ADA); S-(4-nitrobenzyl)-6-thioinosine (NBTI) 10 μM and dipyridamole 10 μM were used to inhibit the transport of adenosine and/or inosine. N^6-R-phenyl-2-propyladenosine (R-PIA) 100 μM was used as a synthetic non-metabolizable analogue of adenosine, and nicotinamide 5 mM was used to inhibit poly(ADP-ribose) polymerase (PARP).

HPLC Analysis of Extracellular Purines

Extracellular purines were measured as previously described.[19] 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°C) and resuspended at 1/10 of the original volume to allow the detection of the low concentrations of purinergic compounds found in these cells. Purine content was determined by a reverse-phase HPLC system equipped with a C-18 column (Supelcosil™, Supelco®, 25 cm × 4.6 mm) and UV detector. Elution was carried out over a 25 min period, at a flow rate of 1.2 ml/min, using a linear gradient from 100% buffer A (KH_2PO_4 60 mM and tetrabutylammonium chloride 5 mM, pH 6.0) to 100% buffer B (buffer A 70% plus methanol 30%). Internal samples were used for identification.

Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS), widely adopted as an index of lipid peroxidation,[29] were measured. After assay, the reaction was stopped by removing the incubation medium. The cells were scraped off, mixed with ice-cold Tris–HCl 15 mM (pH 7.4) and an equal volume of 40% trichloroacetic acid (TCA), followed by addition of 0.67% TBA. Samples were then heated in a boiling water bath for 25 min. After cooling, they were centrifuged (750 *g*/10 min) and the absorbance of the supernatant was read at 535 nm. An absorption coefficient of $1.56 \times 10^5\,M^{-1}\,cm^{-1}$ was used to calculate the amount of TBARS.

Cell Viability Measurements

Lactate dehydrogenase (LDH) activity in the incubation medium was measured at the end of the experimental procedure with a kit (Sigma). Extracellular LDH activity of controls and treatments was compared to the enzymatic activity measured in a homogenate of cells lysed with 1% Triton X. Estimation of trypan blue-excluding cells was also used as an index of viability.

MTT Assay

The MTT colorimetric assay was used to estimate mitochondrial viability, as described by Carmichael *et al.*[30] This method is based on the ability of viable mitochondria 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 in phenol red-free HBSS in the dark) was added to the medium in the wells at a final concentration of 0.2 mg/ml. The cells were left for 45 min at 34°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).

ATP Measurements

The cellular ATP content of Sertoli cells was determined by the well-established luciferin–luciferase method.[31] After assay, cells were washed and the ATP content was extracted with 2% perchloric acid (PCA). Samples were neutralized with NaOH and diluted 200-fold in Tris 10 mM (pH 7.4). An aliquot of this cell extract (60 μl) was mixed with 300 μl of luciferin–luciferase solution (2 mg/ml) and the bioluminescence produced by the reaction with ATP was counted in a luminometer.

Protein Quantification

Protein content was measured as described by Lowry *et al.*[32] and results were standardized against the protein content.

Statistical Analysis

Extracellular purines were measured in at least three separate replicates for each experiment and the mean and standard error calculated. Statistical analysis was performed on the raw data with the ANOVA, with Duncan's *post hoc* test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Selective HPLC analysis showed that addition of H_2O_2 to the incubation medium increased the concentration of extracellular inosine in a dose-dependent manner (Fig. 1a). Hypoxanthine and xanthine were also augmented, although to a lesser extent. These changes were detectable 5 min after addition of hydrogen peroxide, even at the lowest concentration used (25 μM), and were observed up to 3 h after starting incubation. After this period, purine levels of H_2O_2-treated cells returned to basal values (data not shown). LDH activity in the incubation medium was not detected in these time points, indicating that purines in the incubation medium are not due to cell leakage.

Nucleoside uptake inhibitors, dipyridamole and NBTI, increased the H_2O_2-induced accumulation of extracellular adenosine, inosine and hypoxanthine (Fig. 1b). Addition of adenosine deaminase (ADA) inhibitor, EHNA, reduced the H_2O_2-induced increase in inosine levels to 25% and also increased the levels of adenosine (Fig. 1b), suggesting that

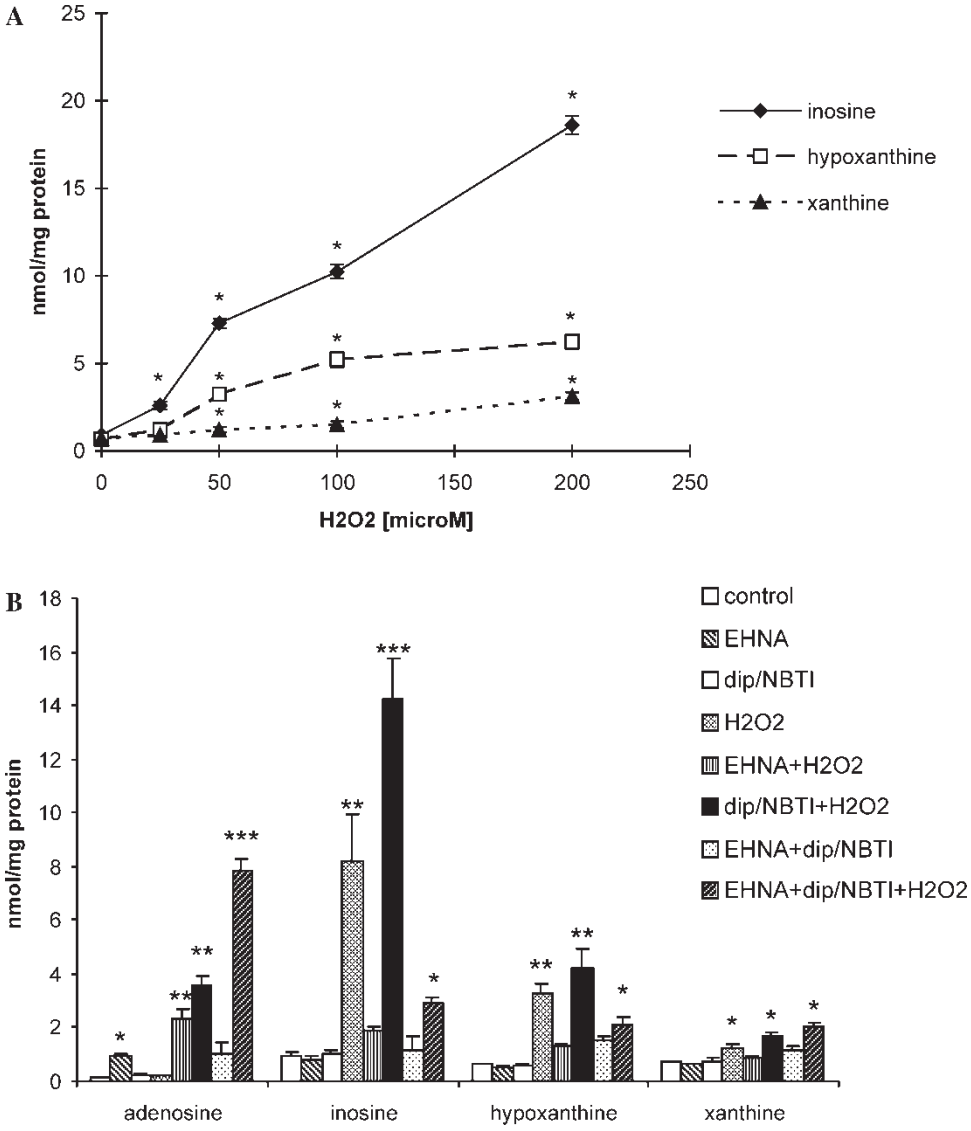

FIGURE 1 *Increased extracellular purines in H_2O_2-treated Sertoli cells.* (A) Cultured Sertoli cells were treated with increasing concentrations of H_2O_2 for 60 min in a humidified 5% CO_2 atmosphere at 34°C. After this period, incubation medium was removed and the purinergic content quantified by HPLC as described in "Materials and Methods Section". (B) Cultures of Sertoli cells were treated with H_2O_2 50 μM for 60 min in the presence of ADA inhibitor EHNA 10 μM, as well as nucleoside uptake inhibitors dipyridamole 10 μM and NBTI 10 μM (dip/NBTI). Representative data from four and three independent experiments, respectively. Each point represents mean ± SEM, $n = 3$. *Different from control. **Different from *group. ***Different from **group.

the increase in inosine levels was due to enhanced ecto-ADA activity. To confirm this hypothesis, we measured the activity of ecto-ADA in the presence of H_2O_2. Figure 2 shows that the rate of degradation of exogenous added adenosine (25 μM) by ecto-ADA is increased in the presence of H_2O_2 50 μM. Co-treatment with EHNA inhibited ecto-ADA activity in both treatments (Fig. 2). Although we have previously reported that Sertoli cells are able to produce inosine from ATP ectonucleotidase action,[19,27] no difference on the rate of degradation of exogenous added ATP (25 μM) by Sertoli cells exposed to H_2O_2 was observed (data not shown).

Cellular levels of TBARS were increased after 60 min incubation with H_2O_2 (50 μM), and the presence of EHNA increased this effect (Fig. 3a). Addition of exogenous inosine restored lipoperoxidation levels to control values. The protective effect of inosine, however, was not observed when nucleoside uptake was blocked by dipyridamole/NBTI. These data indicate that inosine exerts a protective effect against H_2O_2-induced lipoperoxidation via an intracellular mechanism. Although adenosine administration also protected cells against lipoperoxidation, inhibition of ecto-ADA by EHNA abolished this effect, indicating that adenosine-mediated protection was due to its conversion to inosine (Fig. 3b). R-PIA, a synthetic analogue of adenosine which can not be converted to inosine, also had no protective effect against lipoperoxidation (Fig. 3b).

There was a decrease in mitochondrial viability 24 h after H_2O_2 (50 μM) treatment, detected by

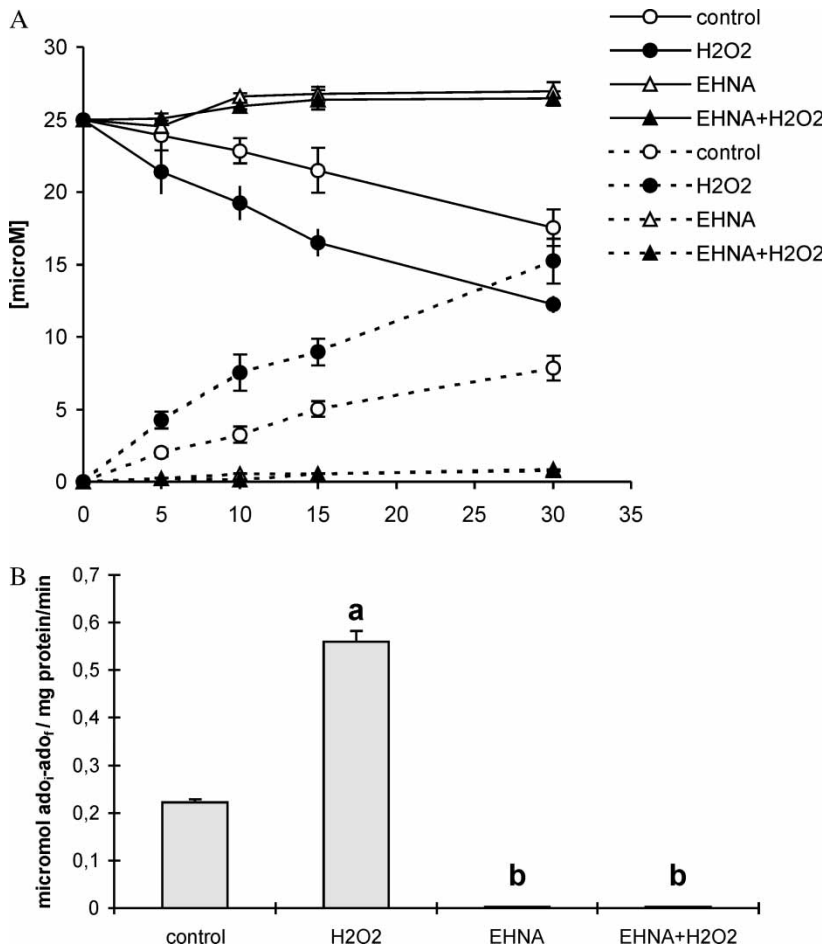

FIGURE 2 *Ecto-ADA activity in H_2O_2-treated Sertoli cells.* (A) The degradation of exogenous added adenosine 25 μM (continuous line) and the concomitant production of inosine (dotted line) in the absence (blank circles) or the presence (filled circles) of H_2O_2 50 μM were measured by HPLC. The effect of EHNA 10 μM with (filled triangles) or without (blank triangles) H_2O_2 was also evaluated. (B) Data shown in A were used to calculate ecto-ADA activity, expressed as micromoles of adenosine degraded/mg protein/minute. EHNA (10 μM) completely inhibited enzymatic activity in both treatments. Representative data from three independent experiments. Each point represents mean ± SEM, $n = 3$. Letters indicate statistical differences between groups.

the MTT assay (Fig. 4a). Administration of inosine prevented H_2O_2-induced damage, restoring mitochondrial viability to levels near to control values. EHNA treatment slightly decreased mitochondrial function, but did not appear to increase the effect of H_2O_2. Inhibition of nucleoside uptake also abolished the protective effect of inosine on H_2O_2-induced damage. Adenosine administration also decreased the effect of H_2O_2, but this effect was not observed in the presence of EHNA or using R-PIA (Fig. 4b). LDH activity in the incubation medium was increased 48 h after H_2O_2 treatment. Similarly, inosine prevented cell leakage in the presence of H_2O_2 (Fig. 5a). Interestingly, EHNA or dip/NBTI alone were able to decrease cellular viability, although to a small extent. Co-treatment with H_2O_2 and EHNA enhanced cellular injury, and dipyridamole/NBTI abolished the protective effect of inosine in H_2O_2-treated cells. Trypan blue exclusion tests confirmed the data observed in the LDH assay (Fig. 5b). As seen in MTT assay, administration of adenosine had a protective effect in both LDH and trypan blue assays, but this effect was also abolished by EHNA (data not shown).

To better understand the mechanism of inosine-mediated protection, we tested the effect of nicotinamide, a well-described and widely adopted inhibitor of poly(ADP-ribose) polymerase (PARP), once it was reported that inosine can prevent oxidant-induced necrosis by inhibiting PARP activation.[33] Nicotinamide had no effect on H_2O_2-induced lipoperoxidation, and the effect of inosine against lipoperoxidation was not altered by the PARP inhibitor (Fig. 6a), suggesting that PARP is not involved on the effect of inosine against H_2O_2-induced lipoperoxidation. Indeed, no effect of nicotinamide treatment on mitochondrial viability was detected by MTT assay (Fig. 6b), and although PARP inhibition alone prevented H_2O_2-induced LDH leakage, it also had no effect on the inosine-mediated cytoprotection (Fig. 6c).

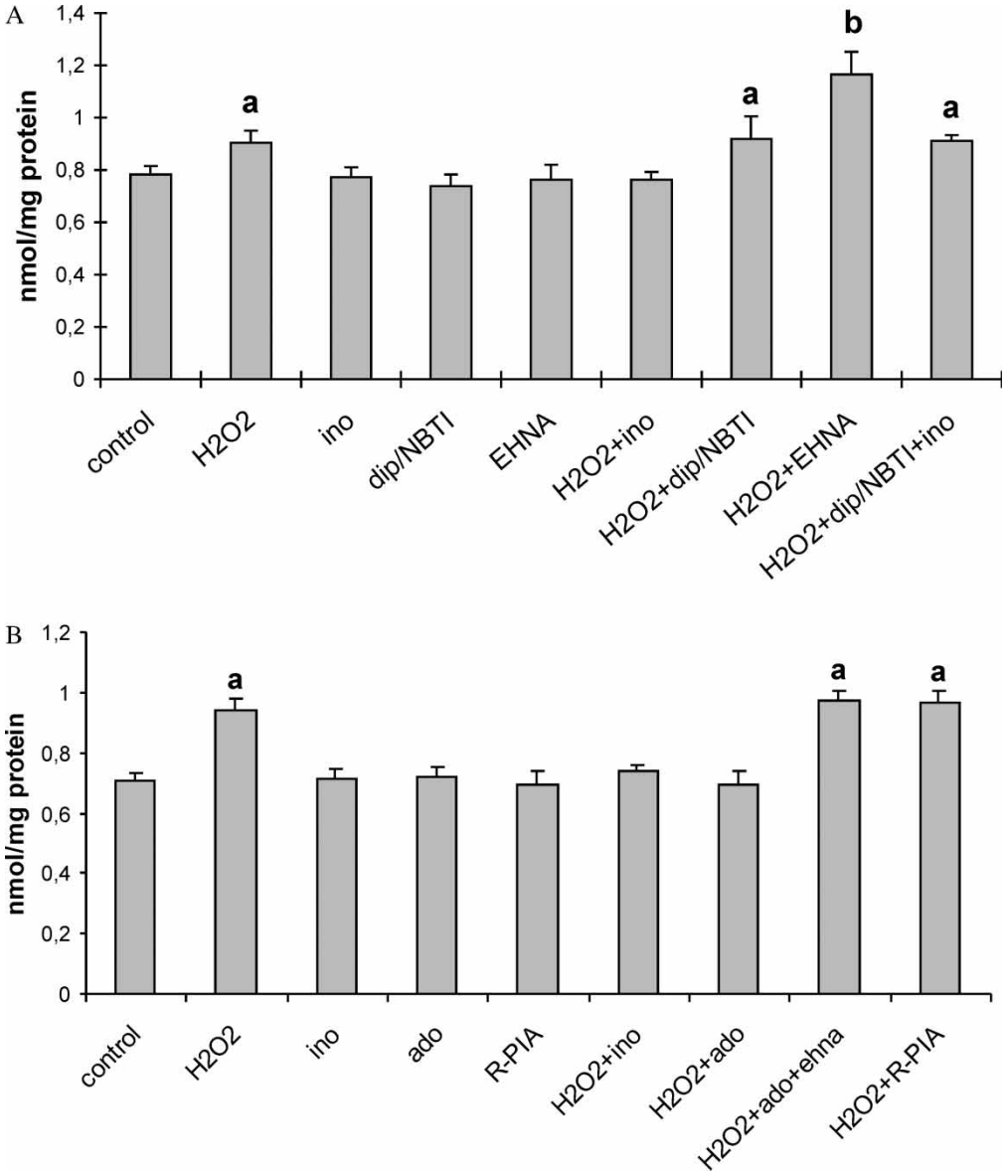

FIGURE 3 *Increased formation of thio barbituric acid-reactive substances (TBARS) by H_2O_2-treated Sertoli cells*. Cultured Sertoli cells were treated with H_2O_2 50 μM for 60 min. The effects of inosine 500 μM and either EHNA 10 μM or dipyridamole and NBTI (dip/NBTI) 10 μM (A), as well as adenosine 500 μM in the presence or absence of ecto-ADA inhibitor EHNA and the adenosine analogue R-PIA 100 μM (B) on cell oxidation were evaluated by assessment of TBARS as described in "Materials and Methods Section". Data are from four independent experiments. Each point represents mean ± SEM, $n = 6$. Letters indicate statistical differences between groups.

Some works have shown that oxidant treatment leads to increased ATP consumption and subsequent cellular ATP depletion.[31,34–36] Once it was reported that inosine can improve cell survival during conditions of ATP depletion and diminished ATP production in cells of NS,[37–39] we evaluated the effect of inosine on cellular levels of ATP during H_2O_2 exposure. Figure 7 shows that H_2O_2 induces a decrease (about 20%) in the ATP content of Sertoli cells, and administration of inosine to H_2O_2-treated cells prevented this effect. Nucleoside uptake blockers abolished inosine-mediated ATP preservation.

DISCUSSION

It has been well-documented that extracellular adenosine has protective effects in ischemic stress. Several reports demonstrate that levels of this nucleoside increase in myocardial tissue or the central NS from nanomolar to micromolar concentrations during episodes of ischemia, thus activating A_1 or A_3 receptors and conferring cytoprotection.[for review see 40] Recent reports have shown that the degradation product, inosine, produced by ADA,[37,38] mediates some of the protective actions of adenosine. Inosine improves

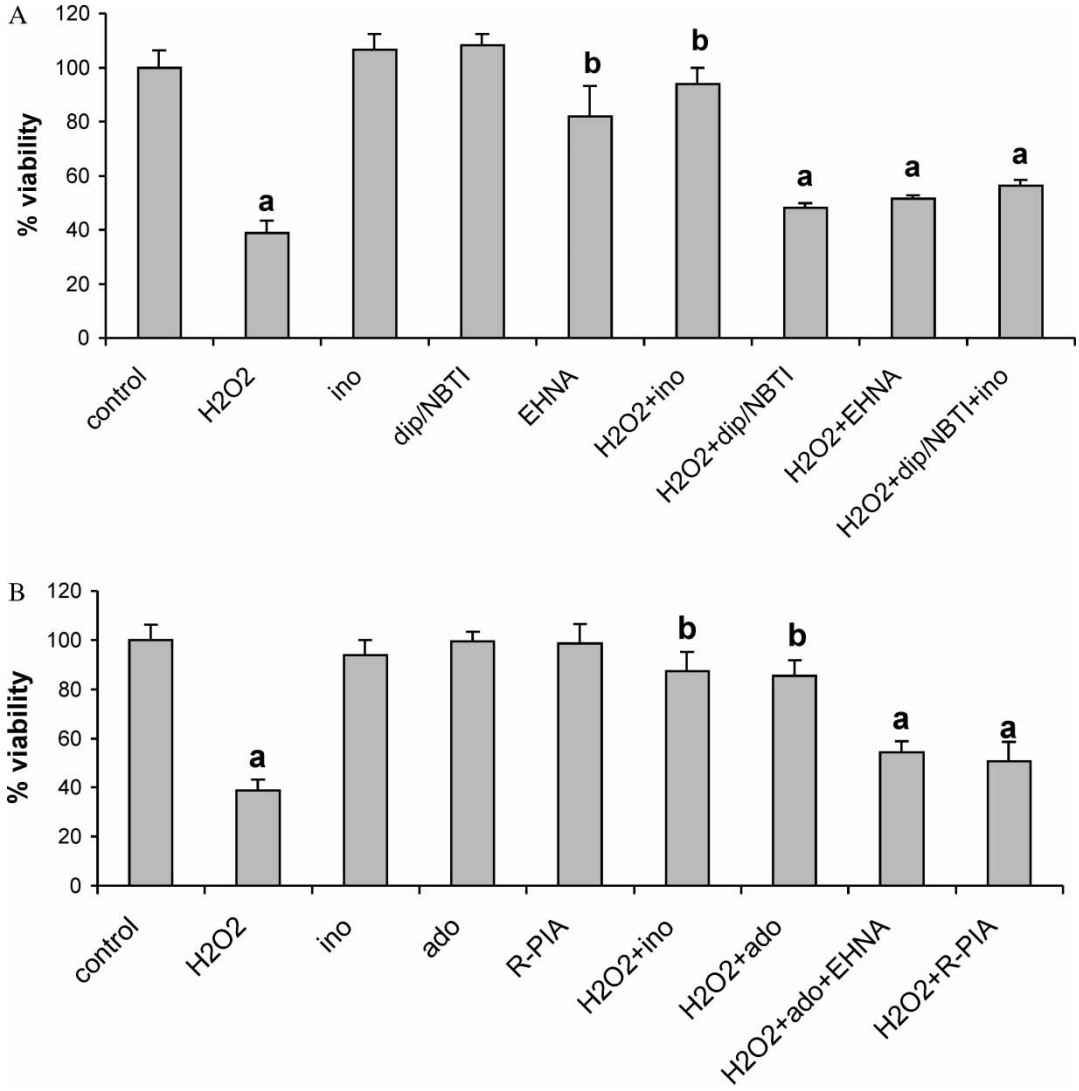

FIGURE 4 *MTT assay.* Cultured Sertoli cells were treated with H_2O_2 50 μM, and the effects of inosine 500 μM and either EHNA 10 μM or dipyridamole 10 μM and NBTI 10 μM (dip/NBTI) on mitochondrial viability were evaluated after 24 h incubation, using the MTT assay, as described in "Materials and Methods Section" (A). The effect of adenosine analogue R-PIA 100 μM, as well as adenosine 500 μM in the presence and absence of EHNA, were also evaluated (B). Data are from three independent experiments. Each point represents mean ± SEM, $n = 4$. Letters indicate statistical differences between groups.

renal function during ischemia[41,42] and removes the harmful effects of ischemia in the liver.[43] It also improves myocardial function during acute left ventricular failure[44,45] and decreases infarct size after coronary occlusion.[46,47]

In this paper, we report for the first time that treatment with H_2O_2 dramatically increases inosine concentrations in the extracellular space of Sertoli cells, and that this nucleoside is able to protect them against H_2O_2-induced lipoperoxidation and cell damage. This modulation of inosine levels by H_2O_2 was shown to be dose-dependent, and concentrations as low as 25 μM hydrogen peroxide readily altered inosine levels (Fig. 1a). We suggest that these modulations occur mainly via activation of ecto-ADA for the following reasons: (1) EHNA markedly inhibited this effect (Fig. 1b); (2) although an increase in the activity of the ecto-enzymes from the purinergic degradation cascade (i.e. ATP → inosine) also could enhances the rate of inosine production, only ecto-ADA activity was found to be augmented (Fig. 2), while no differences on the rates of degradation of exogenous added ATP, ADP and AMP were detected in the presence of H_2O_2 (data not shown). Also, it should be noted that inhibition of ecto-ADA by EHNA with concomitant blockade of adenosine/inosine uptake by dipyridamole/NBTI caused a significant increase in extracellular adenosine levels in H_2O_2-treated cells (Fig. 1b), suggesting that H_2O_2 is increasing the concentration of adenosine in the extracellular environment.

In cerebral and heart ischemia, the dramatic increase in extracellular adenosine observed has been related to the cellular response against ischemic-induced injury, leading to preconditioning[8,10] and cytoprotection.[48] Since H_2O_2 can

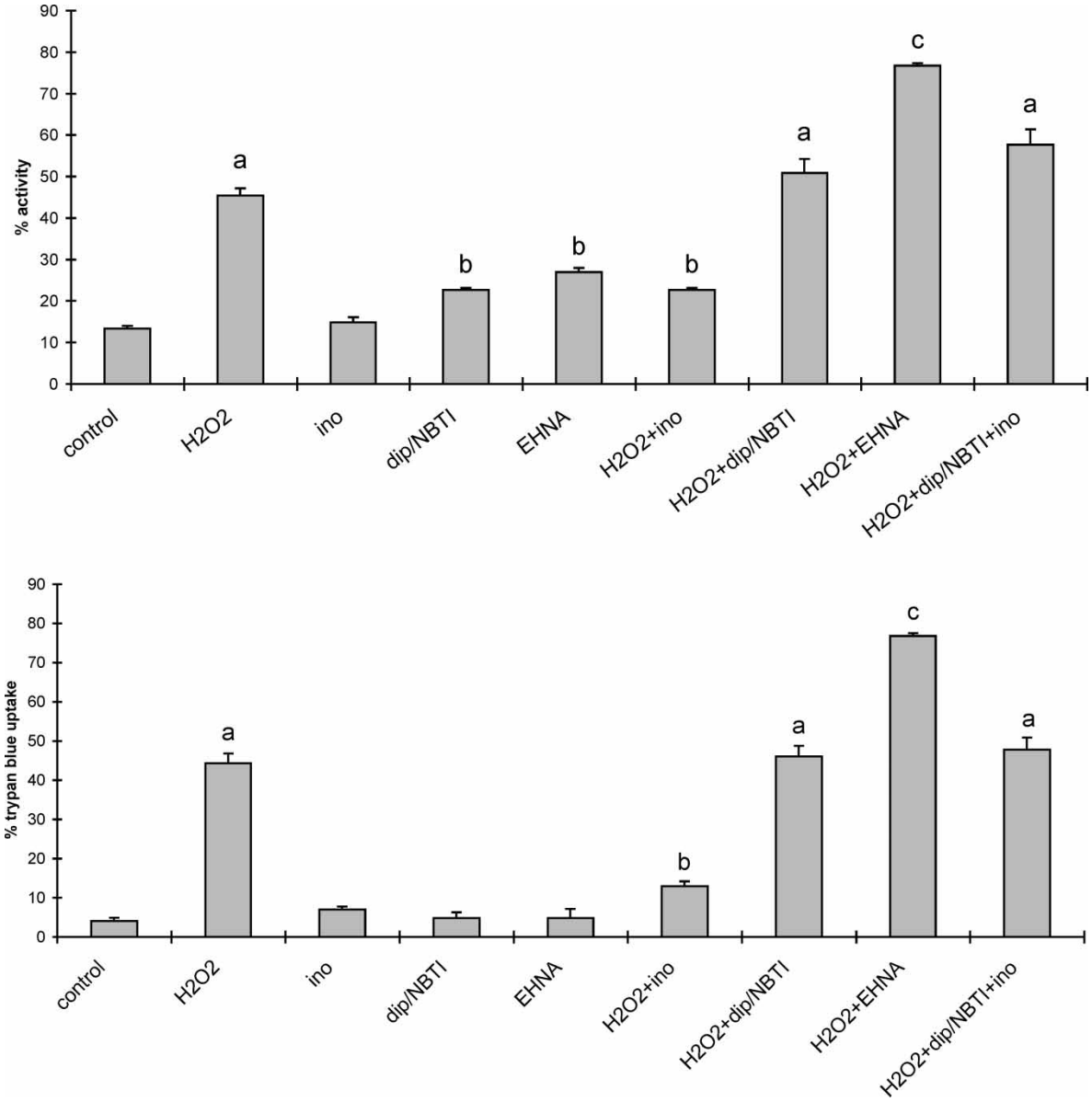

FIGURE 5 *Cellular viability of H_2O_2-treated Sertoli cells*. Cultured Sertoli cells were treated with H_2O_2 50 μM, and the effects of inosine 500 μM and either EHNA 10 μM or dipyridamole 10 μM and NBTI 10 μM (dip/NBTI) on cell viability was estimated after 48 h, using assessment of LDH activity in the incubation medium (A) and the trypan blue exclusion test (B), as described in "Materials and Methods Section". Data are presented as relative to total LDH activity (100% activity: 0,396 μmol NADH/mg protein/minute) or total trypan blue uptake in cells lysed with 1% Triton X. Data are from three independent experiments. Each point represents mean ± SEM, $n = 4$. Letters indicate statistical differences between groups.

modulate inosine levels in Sertoli cells in a similar way, we tested the hypothesis that this nucleoside may be involved in the response of these cells to H_2O_2-induced oxidative stress. The TBARS assay showed that addition of exogenous inosine completely inhibited lipoperoxidation (Fig. 3). Similar results were observed with MTT (Fig. 4) and LDH/trypan exclusion assays (Fig. 5), where inosine administration prevented the cytotoxic effects of H_2O_2, preserving mitochondrial function and maintaining cellular integrity. ADA seems to play an important role in this respect, since EHNA enhanced both the H_2O_2-induced formation of TBARS (Fig. 3a) and the decrease in cell viability (Fig. 5a). Also, EHNA abolished the protective effect obtained by the addition of adenosine (Figs. 3b, 4b and 5b), indicating that this nucleoside has to be converted into inosine to protect cells against H_2O_2. These data reinforce the importance of ADA in the cellular response to H_2O_2.

Although inosine seems to be mainly produced in the extracellular space, our data indicates that this nucleoside acts preferentially via an intracellular mechanism. The triggering of A_3 receptors can activate the antioxidant defence system in RBL-2H3 cells,[5] but, although inosine has been reported to

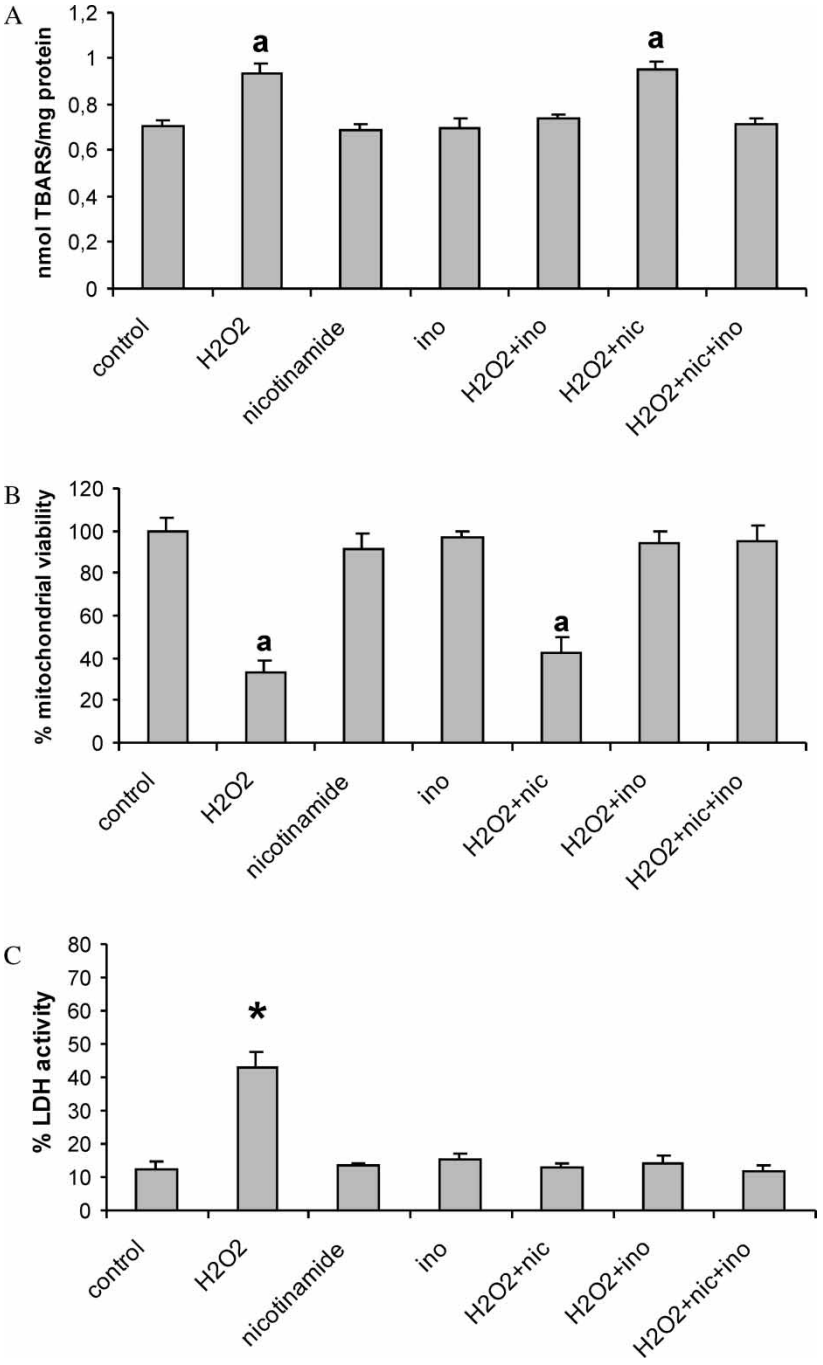

FIGURE 6 *Effect of PARP inhibition on inosine-mediated protection against H_2O_2.* Cultured Sertoli cells were treated with H_2O_2 50 μM in the presence or the absence of nicotinamide 5 mM to evaluate the effect of PARP inhibition on inosine-mediated protection. Lipoperoxidation (A) was evaluated by TBARS assay, mitochondrial viability was assessed by MTT assay (B) and cellular integrity by LDH release (C). Each assay was made as described above. Letters indicate statistical differences between groups. *Different from control.

activate A_3 receptors,[4,49] these receptors were not detected in Sertoli cells.[18] In addition, assays carried out in the presence of the nucleoside uptake inhibitors, dipyridamole and NBTI, showed that the protective effect of inosine is abolished when its internalization is blocked, suggesting that its action is not mediated by triggering of purinoceptors in Sertoli cells. Indeed, the H_2O_2-induced accumulation of inosine in the extracellular space is strongly enhanced in the presence of dipyridamole/NBTI (Fig. 1b). Taking all these results into account, it seems likely that the increased inosine produced by H_2O_2 is actively transported inside Sertoli cells during their exposure to H_2O_2.

In fact, endogenous action of inosine has already been described. It was demonstrated that inosine and, especially, hypoxanthine, in milimolar concentrations, inhibit PARP activation, conferring

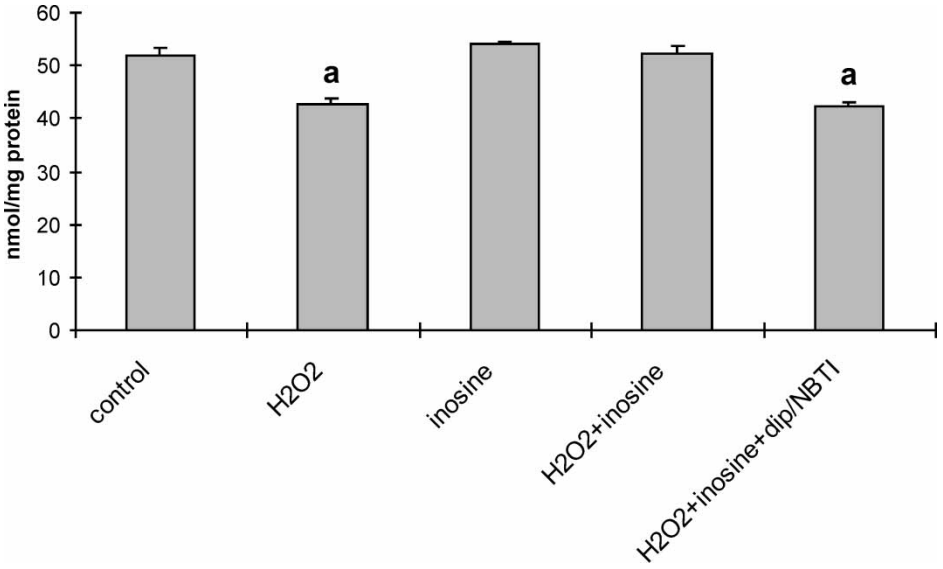

FIGURE 7 *Inosine-mediated preservation of cellular ATP content during H_2O_2 exposure*. Cultured Sertoli cells were treated with H_2O_2 50 μM, and the effect of inosine 500 μM on cellular levels of ATP was evaluated by the luciferin–luciferase assay, as described in "Materials and Methods Section". Each point represents mean ± SEM, $n = 3$. Letter indicate statistical difference between groups.

cytoprotection upon macrophages exposed to peroxynitrite.[38] We tested the effect of inosine on lipoperoxidation and cell survival in the presence of a PARP inhibitor. No difference on the effect of inosine against H_2O_2-induced lipoperoxidation was detected (Fig. 6a), and although PARP inhibition alone conferred cytoprotection to cells treated with H_2O_2 (in agreement with data reported by many authors[31,34–36]), nicotinamide treatment not altered the improvement on cell survival conferred by inosine (Fig. 6c).

On the other hand, inosine was able to preserve cellular ATP levels during H_2O_2 exposure (Fig. 7). This action of inosine is in agreement with data reporting the cytoprotective effects of this nucleoside in cells of the NS subjected to glucose-oxygen deprivation.[37–39] Although the exact mechanism of inosine protection was not elucidated, the authors suggested that during glucose deprivation inosine could confer cytoprotection by ATP preservation, since its degradation produces a ribose-1-phosphate moiety. This molecule can be converted to ribose-5-phosphate by an isomutase and then enter the glycolytic pathway via the pentose phosphate pathway, supplying metabolic fuel to the cells via anaerobic glycolysis. Thus, it is possible that the mechanism of inosine-mediated protection against H_2O_2 in Sertoli cells is similar to that proposed for cells of NS on energy depletion, and further studies will address this issue.

Acknowledgements

We would like to thank our dear colleague Ana Paula Horn for helpful advice and assistance on MTT assay, and Dr Felipe Dal-Pizzol for suggestions and critical review of the manuscript. CAPES, CNPq, FAPERGS and PROPESQ-UFRGS supported this work.

References

[1] Dubyak, G.R. and El-Moatassim, C. (1993) "Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides", *Am. J. Physiol.* **265**, C577–C606.
[2] Klinger, M., Freissmuth, M. and Nanoff, C. (2002) "Adenosine receptors: G protein-mediated signalling and the role of accessory proteins", *Cell Signal.* **14**, 99–108.
[3] Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J.M., Morelli, A., Torboli, M., Bolognesi, G. and Baricordi, R. (2000) "Nucleotide receptors: an emerging family of regulatory molecules in blood cells", *Blood* **97**, 587–600.
[4] Tilley, S.L., Wagoner, V.A., Salvatore, C.A., Jacobson, M.A. and Koller, B.H. (2000) "Adenosine and inosine increase cutaneous vasopermeability by activating A_3 receptors on mast cells", *J. Clin. Investig.* **105**, 361–367.
[5] Maggirwar, S.B., Dhanraj, D.N., Somani, S.M. and Ramkunar, V. (1994) "Adenosine acts as an endogenous activator of the cellular antioxidant defense system", *Biochem. Biophys. Res. Commun.* **201**, 508–515.
[6] Hirai, K. and Ashraf, M. (1998) "Modulation of adenosine effects in attenuation of ischemia and reperfusion injury in rat heart", *J. Mol. Cell. Cardiol.* **30**, 1803–1815.
[7] Kitakaze, M., Minamino, T., Node, K., Takashima, S., Funaya, H., Kuzuya, T. and Hori, M. (1999) "Adenosine and cardioprotection in the diseased heart", *Jpn Circ. J.* **63**, 231–243.
[8] Cross, H.R., Murphy, E., Black, R.G., Auchampach, J. and Steenbergen, C. (2002) "Overspression of A(3) adenosine receptors decreases heart rate, preserves energetics, and protects ischemic hearts", *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1562–H1568.
[9] Maddock, H.L., Mocanu, M.M. and Yellon, D.M. (2002) "Adenosine A(3) receptor activation protects the myocardium from reperfusion/reoxygenation injury", *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1307–H1313.
[10] Dana, A., Jonassen, A.K., Yamashita, N. and Yellon, D.M. (2000) "Adenosine A_1 receptor activation induces delayed

preconditioning in rats mediated by manganese superoxide dismutase", *Circulation* **101**, 2841–2848.
[11] Narayan, P., Mentzer, R.M., Jr. and Lasley, R.D. (2001) "Adenosine A_1 receptor activation reduces reactive oxygen species and attenuates stunning in ventricular myocytes", *J. Mol. Cell. Cardiol.* **33**, 121–129.
[12] Nie, Z., Mei, Y., Ford, M., Rybak, L., Marcuzzi, A., Ren, H., Stiles, G.L. and Ramkunar, V. (1998) "Oxidative stress increases A1 adenosine receptor expression by activating nuclear factor kappa B", *Mol. Pharmacol.* **53**, 663–669.
[13] Lee, H.T. and Emala, C.W. (2002) "Adenosine attenuates oxidant injury in human proximal tubular cells via A_1 and A_{2a} adenosine receptors", *Am. J. Physiol. Renal Physiol.* **282**, F844–F852.
[14] Richard, L.F., Dahms, T.E. and Webster, R.O. (1998) "Adenosine prevents permeability increase in oxidant-injured endothelial monolayers", *Am. J. Physiol. Heart Circ. Physiol.* **274**, H35–H42.
[15] Jégou, B. and Sharpe, R.M. (1993) "Paracrine mechanisms in testicular control", In: de Krester, D., ed, *Molecular Biology of the Male Reproductive System* (Academic Press, San Diego), pp 271–310.
[16] Eusebi, F., Grassi, F., Fratamico, G., Dolci, S., Conti, M. and Stefanini, M. (1985) "Cell-to-cell communication in cultured Sertoli cells", *Pflügers Arch.* **404**, 382–384.
[17] Monaco, L., DeManno, D.A., Martin, M.W. and Conti, M. (1988) "Adenosine inhibition of the hormonal response in the Sertoli cell is reversed by Pertussis toxin", *Endocrinology* **122**, 2692–2698.
[18] Rivkees, S.A. (1994) "Localization and characterization of adenosine receptor expression in rat testis", *Endocrinology* **135**, 2307–2313.
[19] Gelain, D.P., Souza, L.F. and Bernard, E.A. (2003) "Extracellular purines from cells of seminiferous tubules", *Mol. Cell. Biochem.* **245**, 1–9.
[20] Pineau, C., Le Margueresse, B., Courtens, J.L. and Jégou, B. (1991) "Study *in vitro* of the phagocytic function of Sertoli cells in the rat", *Cell Tissue Res.* **264**, 589–598.
[21] Babior, B.M. (2000) "Phagocytes and oxidative stress", *Am. J. Med.* **109**, 33–44.
[22] Mazzilli, F., Rossi, T., Marchesini, M., Ronconi, C. and Dondero, F. (1994) "Superoxide anion in human semen related to seminal parameters and clinical aspects", *Fertil. Steril.* **62**, 862–868.
[23] Baker, H.W., Brindle, J., Irvine, D.S. and Aitken, R.J. (1996) "Protective effect of antioxidants on the impairment of sperm mobility by activated polymorphonuclear leukocytes", *Fertil. Steril.* **65**, 411–419.
[24] Storey, B.T. (1997) "Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa", *Mol. Hum. Reprod.* **3**, 203–307.
[25] Yoganathan, T., Eskild, W. and Hansson, V. (1989) "Investigation of detoxification capacity of rat testicular germ cells and Sertoli cells", *Free Radic. Biol. Med.* **7**, 355–359.
[26] Bhat, S.G., Nie, Z. and Ramkunar, V. (1999) "Cisplatin up-regulates adenosine A_1 receptors in rat testes", *Eur. J. Pharmacol.* **382**, 35–43.
[27] Casali, E.A., da Silva, T.R., Gelain, D.P., Kaiser, G.R.R.F., Battastini, A.M.O., Sarkis, J.J.F. and Bernard, E.A. (2001) "Ectonucleotidase activities in Sertoli cells from immature rats", *Braz. J. Med. Biol. Res.* **34**, 1247–1256.
[28] Tung, P.S. and Fritz, I.B. (1984) "Extracellular matrix promotes rat Sertoli cell histotypic expression *in vitro*", *Biol. Reprod.* **30**, 213–229.
[29] Rego, A.C., Duarte, E.P. and Oliveira, C.R. (1996) "Oxidative stress in acidic conditions increases the production of inositol phosphates in chick retinal cells in culture", *Free Radic. Biol. Med.* **20**, 175–187.
[30] Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. and Mitchell, J.B. (1987) "Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing", *Cancer Res.* **47**, 936–942.
[31] Filipovic, D.M., Meng, X. and Reeves, W.B. (1999) "Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK_1 cells", *Am. J. Physiol. Renal Physiol.* **277**, 428–436.
[32] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with folinphenol reagent", *J. Biol. Chem.* **193**, 265–275.
[33] Virág, L. and Szabó, C. (2001) "Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death", *FASEB J.* **15**, 99–107.
[34] Hudak, B.B., Tufariello, J., Sokolowski, J., Maloney, C. and Holm, B.A. (1995) "Inhibition of poly(ADP-ribose) synthetase preserves surfactant synthesis after hydrogen peroxide exposure", *Am. J. Physiol. Lung Cell. Mol. Physiol.* **12**, L59–L64.
[35] Schraufstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G. and Cochrane, C.G. (1986) "Oxidant injury of cells", *J. Clin. Investig.* **77**, 1312–1320.
[36] Schraufstatter, I.U., Hyslop, P.A., Hinshaw, D.B., Spragg, R.G., Sklar, L.A. and Cochrane, C.G. (1986) "Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase", *Proc. Natl Acad. Sci. USA* **83**, 4908–4912.
[37] Haun, S.E., Segeleon, J.E., Trapp, V.L., Clotz, M.A. and Horrocks, L.A. (1996) "Inosine mediates the protective effect of adenosine in rat astrocyte cultures subjected to combined glucose-oxygen deprivation", *J. Neurochem.* **67**, 2051–2059.
[38] Jurkowitz, M.S., Litsky, M.L., Browning, M.J. and Hohl, C.M. (1998) "Adenosine, inosine and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation", *J. Neurochem.* **71**, 535–548.
[39] Litsky, M.L., Hohl, C.M., Lucas, J.H. and Jurkowitz, M.S. (1999) "Inosine and guanosine preserve neuronal and glial cell viability in mouse spinal cord cultures during chemical hypoxia", *Brain Res.* **821**, 426–432.
[40] Ramkunar, V., Hallam, D.M. and Nie, Z. (2001) "Adenosine, oxidative stress and cytoprotection", *Jpn J. Pharmacol.* **86**, 0265–0274.
[41] Fernando, A.R., Armstrong, D.M., Griffiths, J.R., Hendry, W.F., O'Donoghue, E.P., Perrett, D., Ward, J.P. and Wickham, J.E. (1976) "Enhanced preservation of the ischaemic kidney with inosine", *Lancet* **1**, 555–557.
[42] De Rougemont, D.F., Brunner, F.P., Torhorst, J., Wunderlich, P.F. and Thiel, G. (1982) "Superficial nephron obstruction and medullary congestion after ischemic injury: effect of protective treatments", *Nephron* **31**, 310–320.
[43] Tilser, I., Martinkova, J. and Chladek, J. (1993) "The effect of metipranolol and inosine on total hepatic ischemia of rats *in vivo*", *Sb. Ved. Pr. Lek. Fak. Karlovy Univerzity Hradci Kralove* **36**, 25–29.
[44] Smiseth, O.A. (1983) "Inosine infusion in dogs with acute ischaemic left ventricular failure: favorable effects on myocardial performance and metabolism", *Cardiovasc. Res.* **17**, 192–199.
[45] Woolard, K.V., Kingaby, R.O., Lab, M.J., Cole, A.W. and Palmer, T.N. (1981) "Inosine as a selective ionotropic agent on ischaemic myocardium?", *Cardiovasc. Res.* **15**, 659–667.
[46] Devous, M.D. and Jones, C.E. (1979) "Effect of inosine on ventricular regional perfusion and infarct size after coronary occlusion", *Cardiology* **64**, 149–161.
[47] Czarnecki, W. and Herbaczynska-Cedro, K. (1982) "The influence of inosine on the size of myocardial ischemia and myocardial metabolism in the pig", *Clin. Physiol.* **2**, 189–197.
[48] Rudolphi, K.A., Schubert, P., Parkinson, F.E. and Fredholm, B.B. (1992) "Neuroprotective role of adenosine in cerebral ischemia", *Trends Pharmacol. Sci.* **13**, 439–445.
[49] Jin, X., Shepperd, R.K., Duling, B.R. and Linden, J. (1997) "Inosine binds to A_3 receptors and stimulates mast cell degranulation", *J. Clin. Investig.* **100**, 2849–2857.