

# Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death

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**ABSTRACT** Purines such as adenosine, inosine, and hypoxanthine are known to have potent antiinflammatory effects. These effects generally are believed to be mediated by cell surface adenosine receptors. Here we provide evidence that purines protect against oxidant-induced cell injury by inhibiting the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Upon binding to broken DNA, PARP cleaves NAD<sup>+</sup> into nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins such as histones and PARP itself. Overactivation of PARP depletes cellular NAD<sup>+</sup> and ATP stores and causes necrotic cell death. We have identified some purines (hypoxanthine, inosine, and adenosine) as potential endogenous PARP inhibitors. We have found that purines (hypoxanthine > inosine > adenosine) dose-dependently inhibited PARP activation in peroxynitrite-treated macrophages and also inhibited the activity of the purified PARP enzyme. Consistently with their PARP inhibitory effects, the purines also protected interferon  $\gamma$  + endotoxin (IFN/LPS)-stimulated RAW macrophages from the inhibition of mitochondrial respiration and inhibited nitrite production from IFN/LPS-stimulated macrophages. We have selected hypoxanthine as the most potent cytoprotective agent and PARP inhibitor among the three purine compounds, and investigated the mechanism of its cytoprotective effect. We have found that hypoxanthine protects thymocytes from death induced by the cytotoxic oxidant peroxynitrite. In line with the PARP inhibitory effect of purines, hypoxanthine has prevented necrotic cell death while increasing caspase activity and DNA fragmentation. As previously shown with other PARP inhibitors, hypoxanthine acted proximal to mitochondrial alterations as hypoxanthine inhibited the peroxynitrite-induced mitochondrial depolarization and secondary superoxide production. Our data imply that purines may serve as endogenous PARP inhibitors. We propose that, by affecting PARP activation, purines may modulate the pattern of cell death during shock, inflammation, and reperfusion injury.—Virág, L., Szabó, C. Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death. *FASEB J.* 15, 99–107 (2001)

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DURING ISCHEMIA, CELLULAR ATP is degraded into AMP, adenosine, inosine, and hypoxanthine resulting

in the massive accumulation of these metabolites in tissues and body fluids (1–4). An increased turnover of purines has also been described in inflammation and shock (5, 6). Adenosine and its primary metabolite inosine are ubiquitous nucleosides that can be released in substantial quantities from ischemic or inflamed tissue (1–4, 7, 8). Adenosine is generally considered to act primarily through cell surface adenosine receptors (A1, A2A, A2B, and A3) (9) and is considered a regulator of numerous physiological and pathophysiological events in multiple tissues (10–18). Adenosine and its analogs can alter the course of a variety of inflammatory diseases such as rheumatoid arthritis, nephritis, and uveitis (10–21). Inosine was generally considered an inactive metabolite. However, recently we and others have demonstrated that inosine can also exert antiinflammatory effects (22–24). Very limited information is available on the pharmacological effects or pathophysiological significance of hypoxanthine, the purine base that is cleaved off from inosine. Elevation of hypoxanthine levels in plasma, cerebrospinal fluid, and urine reflects the severity of tissue injury so well that determination of hypoxanthine in body fluids has been proposed to be a good clinical marker of ischemic and inflammatory diseases (25–30). Hypoxanthine may have a dual role in ischemia and inflammation (31): it may promote tissue injury by serving as substrate for the xanthine oxidase-mediated generation of superoxide radical and thus contribute to tissue injury (32–36) and may also contribute to cell recovery after tissue injury by being used for the regeneration of ATP (31).

Poly (ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme of eukaryotic cells that has been implicated in the response to DNA injury and oxidant-induced cell death (37–40). PARP is a nuclear nick sensor enzyme that becomes activated in response to DNA damage (37). Activated PARP cleaves NAD<sup>+</sup> into nicotinamide and ADP-ribose, and catalyzes the latter on nuclear acceptor proteins such as histones and PARP itself (37). Excessive activation of PARP depletes the cellular NAD<sup>+</sup> and ATP pools and causes necrotic cell death (38–40). PARP has also been implicated in the transcriptional regulation of inflammatory media-

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tors such as inducible nitric oxide synthase (iNOS) (41–43). PARP inhibitors provided remarkable protection from tissue damage, organ dysfunction, and lethality in various forms of reperfusion injury, inflammation, and shock (39, 41, 44, 45).

Although the main effect of purines is mediated via cell surface purine receptors, we hypothesized that purines may also exert cytoprotective effects by interfering with the PARP activation pathway. Our assumption was based on the structural similarity of hypoxanthine, inosine, and adenosine to part of NAD<sup>+</sup>, the substrate of PARP. The first aim of the current study was to investigate whether purines affect oxidant-induced cell death. An additional aim was to investigate whether selected purines are capable of suppressing the catalytic activity of PARP. The potent cytotoxic oxidant peroxynitrite (the reaction product of nitric oxide and superoxide) was used to trigger cell death in these studies because 1) this species is known to be produced in various forms of shock and inflammation; 2) it is responsible for some of the oxidant injury in these conditions; and 3) it is a potent trigger of DNA single strand breakage and PARP activation *in vitro* and *in vivo* (40, 41, 46, 47).

The results of the current study demonstrate that certain purines (chiefly, hypoxanthine) are relatively potent inhibitors of PARP in intact cells and markedly affect oxidant-mediated cell death. Purine-mediated inhibition of PARP may be a novel mechanism whereby purines affect cell death and organ injury in various pathophysiological conditions.

## MATERIALS AND METHODS

### Cells and peroxynitrite treatment

RAW mouse macrophages were cultured in Dulbecco's modified Eagle's medium with high glucose and supplemented with 10% fetal bovine serum. Cells were used at 80% confluence in 96-well or 6-well plates. For experiments involving thymocytes, C57/BL6 mice were used. Animals received food and water *ad libitum* and lighting was maintained on a 12 h cycle. Thymi from 3- to 4-wk-old male mice were aseptically removed and placed into ice-cold RPMI media supplemented with 10% v v<sup>-1</sup> fetal calf serum, 10 mM glutamine, 10 mM HEPES, 100 U ml<sup>-1</sup> penicillin, 100 μg ml<sup>-1</sup> streptomycin. Single cell suspensions were prepared by sieving the organs through a stainless wire mesh. Cells isolated in this way were routinely 95% viable, as assessed by trypan blue exclusion assay. Thymocytes (10<sup>6</sup> cells in 0.5 ml medium) were seeded in 24-well plates.

Peroxyntirite was diluted in phosphate-buffered saline (PBS) (pH 11.0) and added to the cells in a bolus of 50 μl. Cells were then incubated for various times (RAW cells: 20 min for PARP assay, 1 or 24 h for MTT assay; thymocytes: 20 min for PARP assay, 3 h for the measurement of mitochondrial parameters, 4 h for propidium iodide staining, or 6 h for DNA fragmentation and caspase activation). Decomposed peroxynitrite (incubated for 30 min at pH 7.0) served as control and failed to influence any of the parameters studied (see also ref 48). In the RAW macrophages, bacterial lipopolysaccharide (10 μg/ml) and murine interferon γ (50

U/ml) were used to stimulate peroxynitrite generation from endogenous sources. At 24 h, mitochondrial respiration was measured as described below. Nitrite/nitrate production, an indicator of NO synthesis, was measured in the supernatant, as described previously (41). First, nitrate in the culture medium was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 2 h. After 2 h, nitrite concentration in the samples was measured by the Griess reaction by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of conditioned medium. The optical density at 550 nm (OD<sub>550</sub>) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, Calif.). Nitrate concentrations were calculated by comparison with OD<sub>550</sub> of standard solutions of sodium nitrate prepared in culture medium. Purines or other test compounds (up to 3 mM) did not scavenge nitrite or nitrate and did not interfere with the activity of nitrate reductase.

### Measurement of cellular PARP activity

RAW cells cultured in 6-well plates or thymocytes (10<sup>7</sup> cells in 1 ml culture medium) were treated with peroxynitrite (750 μM). After 20 min cells were spun, medium was aspirated, and cells were resuspended in 0.5 ml assay buffer (56 mM HEPES-pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.01% w v<sup>-1</sup> digitonin, and 0.125 μM NAD<sup>+</sup> and 0.5 μCi ml<sup>-1</sup> <sup>3</sup>H-NAD<sup>+</sup>). PARP activity was then measured as described previously (48). Briefly, after incubation (10 min at 37°C), 200 μl ice-cold 50% w v<sup>-1</sup> trichloroacetic acid (TCA) was added and samples incubated for 4 h at 4°C. Samples were spun (10,000 g, 10 min), then pellets were washed twice with ice-cold 5% w v<sup>-1</sup> TCA and solubilized overnight in 250 μl 2% w v<sup>-1</sup> sodium dodecyl sulfate (SDS)/0.1 N NaOH at 37°C. Contents of the tubes were added to 6.5 ml ScintiSafe Plus scintillation liquid (Fisher Scientific, Pittsburgh, Pa.) and radioactivity was determined using a liquid scintillation counter (Wallach, Gaithersburg, Md.).

### Measurement of PARP activity *in vitro*

To assess the direct effect of purines and test compounds on the activity of the purified PARP enzyme, an *in vitro* assay was carried out, as described (49). Briefly, 1 μg of bovine PARP enzyme was added to 200 μl of buffer containing 100 mM Tris: 5 mM MgCl<sub>2</sub>, 1 mM DTT, pH 7.9, in the presence or absence of purines or other test compounds. The mixture was allowed to preincubate at room temperature for 30 min. After preincubation, 10 μl of sonicated DNA (32.5 μg/ml final concentration) and 10 μl histone (4 mg/ml) was added to each tube along with 10 μl <sup>3</sup>H-NAD<sup>+</sup> (16 μCi/μl). Tubes were vortexed, then centrifuged at 10,000 rpm for 10 s, and the reaction was allowed to proceed at 37°C for 15 min. Reactions were stopped by the addition of 200 μl 50% TCA.

Reaction mixtures were kept at -20°C for 30 min. The TCA insoluble precipitate was collected by filtration through 0.2 μm nitrocellulose analytical test filter funnels under vacuum and washed five times with 3 ml of cold 5% TCA. The membranes were counted in 7 ml scintillation vials with Scintisafe mixture for 2 min beta spectrum in a Wallach 1409 scintillation counter.

### Measurement of mitochondrial respiration in macrophages

Cell respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (41). Cells in 96-well

plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100  $\mu$ l). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD<sub>550</sub>.

### Dihydrorhodamine assay

To test whether the purines used in our studies directly scavenge peroxynitrite, the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123 was measured based on the principles of the method previously described (50). Briefly, peroxynitrite (5  $\mu$ M) was added into PBS containing 10  $\mu$ M dihydrorhodamine 123 and different concentrations of purines. After a 10 min incubation at 22°C, the fluorescence of rhodamine 123 was measured using a Shimadzu RF-5301PC fluorometer at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively). In control, reverse-order experiments we have confirmed that the compounds tested neither showed fluorescence at the above wavelengths nor affected rhodamine 123 fluorescence (data not shown).

### Cytochrome *c* oxidation

To use another assay for testing whether the purines used in our studies directly scavenge peroxynitrite, peroxynitrite-dependent oxidation of cytochrome *c*<sup>2+</sup>, was measured as described (50). Cytochrome *c* was reduced by sodium dithionite immediately before use and purified by chromatography on Sephadex G-25 using 100 mM potassium phosphate plus 0.1 mM DTPA, pH 7.2, as the elution buffer. The concentration of cytochrome *c*<sup>2+</sup> was determined spectrophotometrically at 550 nm in the same buffer ( $\epsilon=21 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Cytochrome *c*<sup>2+</sup> oxidation (50  $\mu$ M) yields upon addition of peroxynitrite (25  $\mu$ M initial concentration after mixing) were assessed by incubation of reaction mixtures in 100 mM potassium phosphate plus 0.1 mM DTPA, pH 7.2, at 22°C for 3 min in the absence or presence of purines. Oxidation of cytochrome *c*<sup>2+</sup> was followed at 550 nm using a Shimadzu UV-Z401PC spectrophotometer. In control, reverse-order experiments we have confirmed that the compounds tested do not interfere with the spectrophotometric measurements at the above wavelengths. Moreover, in control experiments we have confirmed that the compounds tested do not reduce cytochrome *c*<sup>3+</sup>.

### Measurement of mitochondrial membrane potential, superoxide production, and cardiolipin content in thymocytes

The mitochondrial membrane potential was quantitated by the flow cytometric analysis of 3,3'-dihexyloxycarbocyanine iodide [DiOC6(3)]-stained cells (51). Intramitochondrial generation of reactive oxygen intermediates was determined by analyzing with flow cytometry the superoxide-induced conversion of the oxidant-sensitive dye dihydroethidium to ethidium (51). Mitochondrial membrane damage was determined by measuring the cardiolipin degradation, as described (51).

### Flow cytometry in thymocytes

Thymocytes were stained with 5  $\mu$ g/ml PI, 40 nM DiOC6(3), 2  $\mu$ M hydroethidine (HE) for 15 min at 37°C, washed once with PBS, and analyzed with a FACS Calibur flow cytometer as described (51). To measure mitochondrial parameters, for-

ward and side scatters were gated on the major population of normal-sized cells. For the cytotoxicity assay, the percentage of PI-positive cells was calculated from the total (ungated) population.

### Detection of internucleosomal DNA fragmentation of thymocytes

Thymocytes were pretreated with purines for 30 min and then treated with peroxynitrite. After 6 h, cells were washed once with cold PBS and pellets were resuspended in sample buffer (10 mM Tris, pH 8.0, 5% v/v glycerol, 0.05% w/v<sup>-</sup> bromophenol blue, 5 mg/ml RNase). DNA fragmentation was detected as described (48). Agarose (2% w/v) was poured on a horizontal gel support. After solidification of the gel, the top part (above the comb) was replaced with 1% w/v agarose containing 2% w/v SDS and 64  $\mu$ g/ml proteinase K. Cells ( $2 \times 10^6$ ) were loaded in 20  $\mu$ l sample buffer. Electrophoresis was carried out at 25 V for 12 h and the gel was stained with 2  $\mu$ g/ml ethidium bromide for 1 h.

### Measurement of caspase 3-like activity

Caspase activity was measured by the cleavage of the fluorogenic tetrapeptide-amino-4-methylcoumarine conjugate (DEVD-AMC) as described (52). Unless otherwise indicated, cells ( $4-10 \times 10^6$ ) were harvested 6 h after peroxynitrite treatment, washed once in PBS, and lysed in a lysis buffer (10 mM HEPES, 0.1% w/v CHAPS, 5 mM dithiothreitol, 2 mM EDTA, 10  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin A, and 1 mM PMSF, pH 7.25) for 10 min on ice. Cell lysates and substrates (50  $\mu$ M) were combined in triplicate in the caspase reaction buffer (100 mM HEPES, 10% w/v sucrose, 5 mM dithiothreitol, 0.1% w/v CHAPS, pH 7.25) in the presence or absence of 10  $\mu$ M of the tetrapeptide caspase 3 inhibitor N-acetyl-aspartyl-glutamyl-valyl-aspartyl-aldehyde (DEVD-CHO) and samples were incubated at 37°C for 60 min. AMC liberation was determined with a Perkin-Elmer fluorometer using 380 nm excitation and 460 nm emission wavelength. Data are given as absolute fluorescence units.

### Materials

Peroxyntirite was a kind gift of Dr. H. Ischiropoulos (Inst. Environmental Medicine, University of Pennsylvania, Philadelphia). 3-Morpholinimidnonimine (SIN-1) was purchased from Calbiochem (San Diego, Calif.). HE, DiOC6(3), and propidium iodide were obtained from Molecular Probes (Eugene, Ore.). The tetrapeptide substrate (DEVD-AMC) and inhibitor (DEVD-CHO) of caspase 3 and the purified PARP enzyme were purchased from Biomol (Plymouth Meeting, Pa.). Proteinase K was obtained from Life Technologies (Grand Island, N.Y.). Tris, magnesium chloride, analytical test filter funnels, and Scintisafe scintillation mixture were from Fisher Scientific. <sup>3</sup>H-NAD<sup>+</sup> was purchased from DuPont NEN (Boston, Mass.). Murine interferon  $\gamma$  was from Genzyme (Cambridge, Mass.). Tissue culture medium and fetal calf serum were from Life Technologies, Inc. Bacterial lipopolysaccharide (*Escherichia coli*, serotype No. 0111:B4) and all other reagents were obtained from Sigma (St. Louis, Mo.).

### Statistical analysis

All values in the figures and text are expressed as mean  $\pm$  standard deviation (SD) of *n* observations; *n*  $\geq$  3. Data sets were examined by analysis of variance and individual group

means were then compared with Bonferroni's *post hoc* test. A *P* value of less than 0.05 was considered statistically significant. When the results are presented as representative gels or flow cytometry analyses, results similar to the ones shown were obtained in at least three different experiments.

## RESULTS

### Purines inhibit peroxynitrite-induced PARP activation

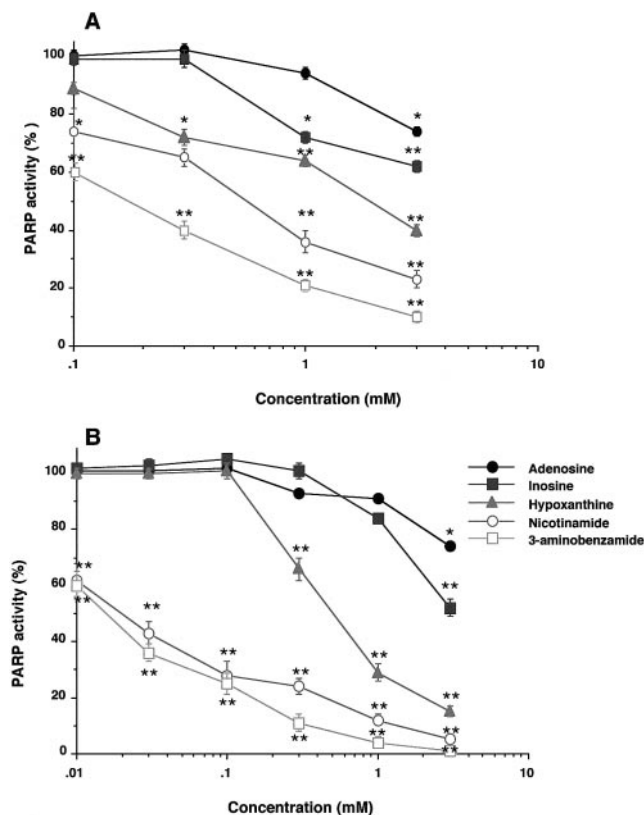
In cultured RAW macrophages, peroxynitrite induced a marked increase in the activity of PARP, which was dose-dependently inhibited by hypoxanthine and inosine (100  $\mu$ M–3 mM). At the highest concentrations used, adenosine exhibited a slight inhibitory effect on PARP. The reference compounds used (the prototypical PARP inhibitors 3-aminobenzamide and nicotinamide) exhibited a dose-dependent inhibitory effect on PARP more potent than that afforded by the purines (Fig. 1A). Nevertheless, the difference between the potency of the most potent purine tested (hypoxanthine) and the least potent 'professional' PARP inhib-

itor used (nicotinamide) was rather small ( $EC_{50}$  values  $\sim$  1 and 2 mM, respectively).

Because differential cell uptake of the purines and PARP inhibitors may influence the potency of PARP inhibition, we also tested the effect of the purines and reference compounds in a cell-free PARP assay. Again, the potency of the purines was hypoxanthine>inosine>adenosine (Fig. 1B), with nicotinamide and 3-aminobenzamide becoming more potent relative to the purines. These results indicate that limited cell uptake markedly reduces the PARP inhibitory potency of 3-aminobenzamide and nicotinamide, but only to a lesser degree in the case of the three purines tested.

### Purines inhibit the suppression of cell viability in immunostimulated macrophages

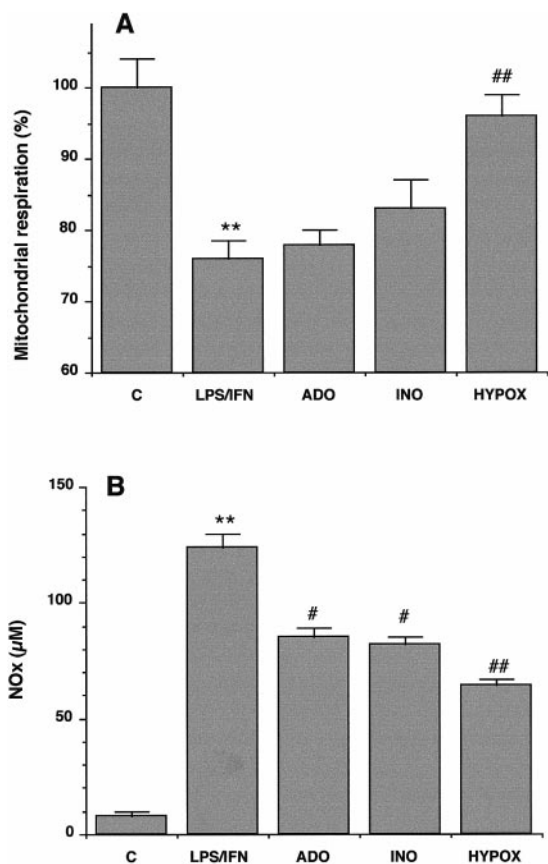
Stimulation of macrophages with bacterial lipopolysaccharide and  $\gamma$ -interferon results in the production of peroxynitrite from endogenous sources, which leads to a decrease in cellular viability that is mediated in part by PARP (47). Here we tested whether adenosine, inosine, or hypoxanthine affect the decrease in cell viability in response to immunostimulation in RAW macrophages. Exposure of the macrophages to bacterial lipopolysaccharide and interferon  $\gamma$  resulted in production of nitrite/nitrate (oxidized products of NO) and a decrease in mitochondrial respiration (Fig. 2). Pretreatment of the cells with hypoxanthine (and, to a lesser extent, inosine), provided significant protection against the suppression of cell viability (Fig. 2A). Furthermore, hypoxanthine (potently) and the other purines tested (weakly) inhibited the production of nitrite/nitrate in response to immunostimulation (Fig. 2B). A similar suppression of immunostimulation-induced NO production and iNOS expression has previously been reported with 3-aminobenzamide, nicotinamide, and other PARP inhibitors, as well as by the PARP negative phenotype (41–43, 53).



**Figure 1.** Purines inhibit PARP activity. RAW macrophages were pretreated for 30 min with the indicated concentrations of purines or PARP inhibitors and then treated with 750  $\mu$ M peroxynitrite. After 20 min, cellular PARP activity was determined as described in Materials and Methods. PARP activity is displayed as percent of peroxynitrite-induced elevation. A) The activity of purines on the purified PARP enzyme was also determined (B). \* $P$ <0.05, \*\* $P$ <0.01 inhibition of PARP activity.

### Purines do not scavenge peroxynitrite

To test whether the purines used exert a direct scavenging effect on peroxynitrite or on the peroxynitrite-generating agent SIN-1 (rather than a direct inhibitory effect on the catalytic activity of PARP), we tested, in two different assays, whether the purines used interfere with peroxynitrite- or SIN-1-mediated oxidations (and thus act as scavengers of peroxynitrite). Adenosine, inosine, or hypoxanthine (at 100  $\mu$ M–3 mM) failed to inhibit the peroxynitrite- and SIN-1-induced dihydrorhodamine 123 oxidation or cytochrome *c* oxidation ( $n$ =9, not shown). The positive control N-acetylcysteine exhibited a dose-dependent inhibitory effect on the peroxynitrite-induced oxidations, with  $EC_{50}$  values of 9  $\mu$ M and 100  $\mu$ M in the dihydrorhodamine and cytochrome assays, respectively ( $n$ =6–9).



**Figure 2.** Effect of purines on mitochondrial respiration and nitrite/nitrate production of immune-stimulated macrophages. Raw macrophages were stimulated for 24 h with LPS + IFN. Immune stimulation resulted in the suppression of mitochondrial respiration, as determined by MTT reduction assay (A). Pretreatments were 3 mM adenosine (ADO), 3 mM inosine (INO), and 3 mM hypoxanthine (HYPOX). \*Significant suppression of mitochondrial respiration (\*\* $P < 0.01$ ); # indicates significant ( $##P < 0.01$ ) protection from the suppression of respiration. The production of nitrite/nitrate by immunostimulated RAW macrophages has also been determined (B).

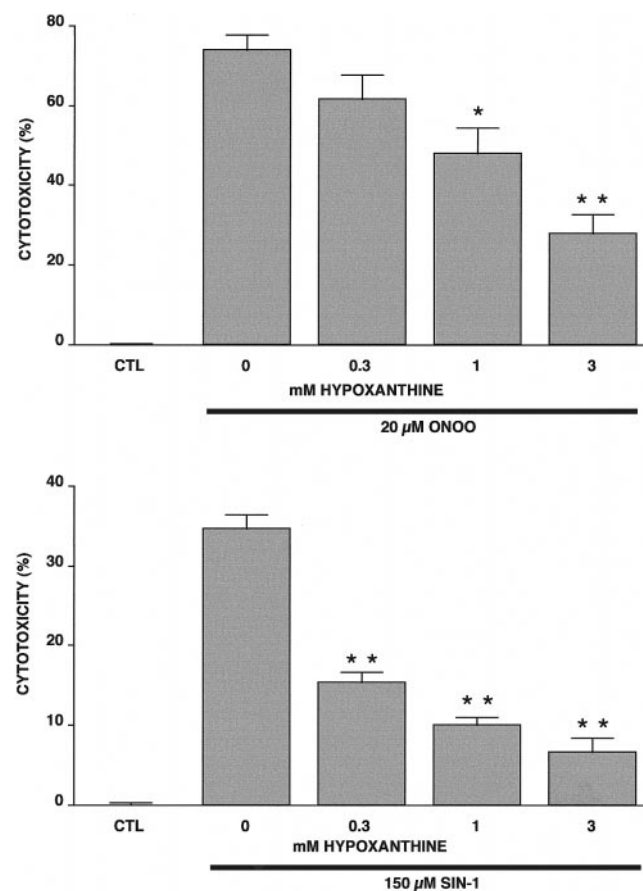
### Effect of hypoxanthine on peroxynitrite-induced thymocyte death and mitochondrial alterations

Since hypoxanthine was the most potent PARP inhibitor from the group of purines tested, we selected this agent for detailed evaluation on the mode and mechanism of peroxynitrite-induced cell death in thymocytes. Authentic peroxynitrite (20  $\mu\text{M}$ ) caused thymocyte death, as assessed by the uptake of propidium iodide (Fig. 3A). Since we have demonstrated previously that PARP activation mediates necrotic thymocyte death in this system (48, 51), we subsequently investigated the effect of hypoxanthine on peroxynitrite-induced necrosis. In line with its PARP inhibitory effect, hypoxanthine (300  $\mu\text{M}$ –3 mM) inhibited peroxynitrite-induced cytotoxicity in a dose-dependent manner (Fig. 3, top panel). In addition to bolus addition of authentic peroxynitrite, we also used SIN-1, a peroxynitrite-generating agent. Hypoxanthine has also provided concentration-dependent protection against SIN-1-induced cytotoxicity (Fig. 3, bottom panel).

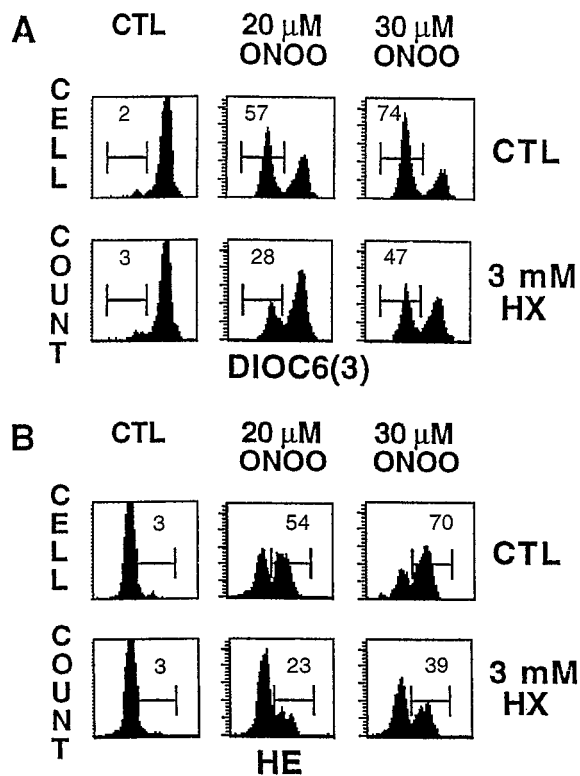
Mitochondria maintain a negative transmembrane potential that dissipates during various forms of cell death, both apoptosis and necrosis. The mitochondrial transmembrane potential can be quantitated by using cationic fluorescent dyes such as DiOC(6)<sub>3</sub>, which are accumulated by the mitochondria. Peroxynitrite-induced thymocyte necrosis is also accompanied by a decrease of the mitochondrial transmembrane potential and increased secondary superoxide production (51). These mitochondrial alterations have also been found to be dependent on PARP activation (51). Consistent with the PARP inhibitory effect of hypoxanthine, the compound reduced the collapse of mitochondrial membrane potential as indicated by DiOC(6)<sub>3</sub> uptake (Fig. 4A). Furthermore, hypoxanthine also inhibited peroxynitrite-induced secondary superoxide production (Fig. 4B).

### Effect of hypoxanthine on the mode of peroxynitrite-induced cell death

We have previously shown that peroxynitrite causes both apoptotic and necrotic cell death (48). Further-



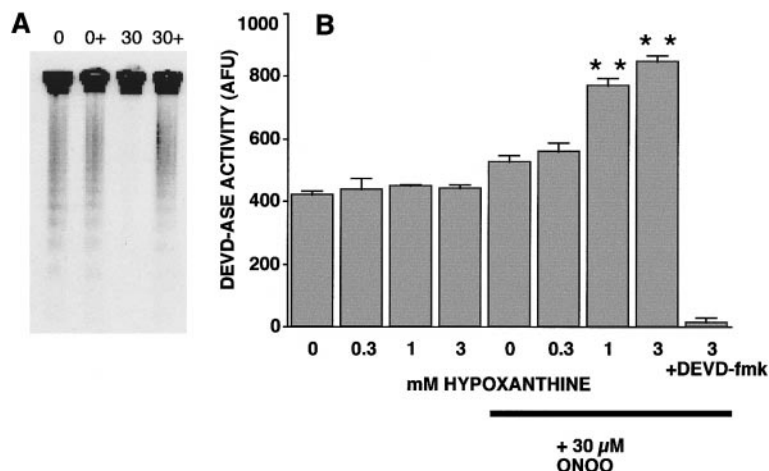
**Figure 3.** Effect of hypoxanthine on peroxynitrite-induced thymocyte death. Thymocytes were pretreated with the indicated amount of hypoxanthine for 30 min and then treated with 20  $\mu\text{M}$  authentic peroxynitrite (A) or 150  $\mu\text{M}$  SIN-1 (B). After 3 h cells were stained with propidium iodide and analyzed by flow cytometry. Means  $\pm$  SD of triplicate samples are shown. Asterisks indicate significant (\* $P < 0.05$ , \*\* $P < 0.01$ ) inhibition of cytotoxicity.



**Figure 4.** Effect of hypoxanthine on mitochondrial membrane depolarization and secondary superoxide production of peroxynitrite-treated thymocytes. Thymocytes were pretreated with 3 mM hypoxanthine for 30 min, then treated with 20 and 30  $\mu\text{M}$  peroxynitrite. After 3 h, cells were stained with DIOC6(3) to measure mitochondrial membrane potential (A) and hydroethidine (HE) to determine superoxide production (B). Data are shown as representative experiments of 3 independent experiments. Numbers indicate percent number of cells displaying decreased mitochondrial membrane potential or increased superoxide production. CTL, control; HX, hypoxanthine; ONOO, peroxynitrite.

more, our group and others have also shown that PARP activation diverts oxidant-induced cell death from the default apoptosis toward necrosis (48, 54, 55). Accordingly, in the presence of PARP inhibitors or in PARP-deficient cells, an increased output of apoptotic parameters such as caspase activation and DNA fragmentation

**Figure 5.** Effect of hypoxanthine on caspase activation and DNA fragmentation in peroxynitrite (ONOO)-treated thymocytes. Thymocytes were pretreated with 3 mM hypoxanthine for 30 min, then treated with 30  $\mu\text{M}$  peroxynitrite. Cells were then incubated for 6 h and analyzed for DNA fragmentation by agarose gel electrophoresis (A) and for caspase activation by DEVD-AMC cleavage (B). A) Numbers indicate the concentration ( $\mu\text{M}$ ) of peroxynitrite used; + = hypoxanthine pretreatment. B) Means  $\pm$  SD of triplicate samples are given and asterisks indicate significantly (\*\* $P < 0.01$ ) increased caspase activity. The last column indicates DEVD-ase activity of 3 mM hypoxanthine + 30  $\mu\text{M}$  peroxynitrite-treated cells measured in the presence of 10  $\mu\text{M}$  caspase inhibitor (DEVD-fmk).



can be observed (48, 54). We have investigated the effect of hypoxanthine on these apoptotic parameters. Hypoxanthine (3 mM) had no effect on the spontaneous DNA fragmentation of thymocytes (Fig. 5A), which reflects the spontaneous apoptosis that thymocytes are known to undergo in culture. Peroxynitrite (20  $\mu\text{M}$ ) inhibited the spontaneous DNA fragmentation of thymocytes, an effect that has previously been demonstrated to be mediated by PARP activation (48). Hypoxanthine (3 mM) prevented the peroxynitrite-induced decrease of oligonucleosomal DNA cleavage (Fig. 5A).

Peroxynitrite-induced cell death is also accompanied by the activation of caspase-3 like proteases, which have been shown to mediate DNA fragmentation (48, 52). Small concentrations of peroxynitrite (10–30  $\mu\text{M}$ ) induce caspase activation, whereas at higher concentrations (40–80  $\mu\text{M}$ ) of peroxynitrite caspase activity declines (49). At the concentration used in the current experiment (30  $\mu\text{M}$ ), peroxynitrite induced a small degree of caspase activation as measured by the cleavage of the fluorochrome-conjugated tetrapeptide substrate DEVD-AMC. In the presence of hypoxanthine, however, significantly higher caspase activity has been detected (Fig. 5B). The effect of hypoxanthine was dose dependent. Taken together, the results in thymocytes demonstrated that hypoxanthine, similar to the prototypical PARP inhibitors 3-aminobenzamide and very similar to the findings obtained in PARP-deficient thymocytes exposed to cytotoxic oxidants (48, 51), dose-dependently suppresses peroxynitrite-induced thymocyte necrosis (and related parameters), increases the fraction of cells exhibiting normal phenotype, but also simultaneously increases apoptotic DNA fragmentation.

## DISCUSSION

### Concentration of hypoxanthine in biological fluids and tissues

The purines studied exerted a cytoprotective and PARP inhibitory effect in the millimolar range. The most pertinent question that determines the potential im-

portance of the current findings is, How do these concentrations relate to the tissue, plasma, or intracellular levels of purines under physiological and pathophysiological conditions? The concentrations of purines, including adenosine, inosine, and hypoxanthine, in various body fluids have been determined by several groups (3, 8, 30, 56, 57). Normal values of hypoxanthine in the serum range between 1 and 11  $\mu\text{M}$  (3). However, increases of serum hypoxanthine concentration by two orders of magnitude have been reported in endotoxemic dogs (58) and during the resuscitation of a newborn baby with cardiac arrest (59). Most studies report normal values between 0 and 5  $\mu\text{M}$  in the cerebrospinal fluid, whereas after severe hypoxia levels up to 700  $\mu\text{M}$  have been found (3). Considering the generally held view that tissue levels of purine metabolites far exceed values measured in bodily fluids, tissue hypoxanthine levels are generally estimated to be in the millimolar range, especially under certain pathophysiological conditions such as shock, ischemia, and inflammation. Indeed, sporadic attempts made to determine tissue levels of hypoxanthine support our estimation. For example, normal hypoxanthine levels in rat heart or in human placenta were found to be 138  $\mu\text{M}$  and 284  $\mu\text{M}$ , respectively (3). In bovine chromaffin cells, intracellular concentrations of 2.1, 3.8, and 1.5 mM have been reported for adenosine, inosine, and hypoxanthine, respectively (60). Thus, it is most likely that millimolar concentrations of hypoxanthine occur *in vivo*, and therefore hypoxanthine may well act as an inhibitor of PARP activation in ischemic or inflamed tissues. This PARP inhibitory and cytoprotective effect of hypoxanthine may be further increased by a possible synergism between inosine, adenosine, and hypoxanthine. A synergistic or additional action is also possible between purines and nicotinamide, another endogenous PARP inhibitor whose tissue level also increases during hypoxia due to increased catabolism of nicotinamide coenzymes (61–62).

### Cytoprotective effect of purines by PARP inhibition

Hypoxanthine, and to a lesser extent inosine and adenosine, prevented cytotoxicity induced either by bolus addition of authentic peroxynitrite, continuous generation of peroxynitrite from SIN-1, or endogenous production of peroxynitrite by activated macrophages. The cytotoxic effect of peroxynitrite has previously been shown to be mediated in part by PARP activation (40, 41, 48, 63). Therefore, we investigated whether or not these selected purines interfere with PARP activation. In the millimolar range, the purines tested inhibited PARP activation in RAW macrophages with a potency comparable to the prototypical 'benchmark' PARP inhibitors 3-aminobenzamide and nicotinamide. The purines tested also exerted inhibitory effect on the purified PARP enzyme. However, in this cell-free assay, purines proved to be considerably weaker inhibitors than nicotinamide or 3-aminobenzamide. The better performance of purines in the cellular PARP assay is

likely to be explained by the superior membrane permeability/cell uptake of purines as compared to 3-aminobenzamide or nicotinamide. We have found a good correlation between PARP inhibitory and cytoprotective potency of purines—hypoxanthine being the most potent and adenosine being the least potent in both assays.

### Effect of purines on NO production

Nitric oxide and NO-derived oxidants such as peroxynitrite are important mediators of various forms of inflammation, shock, and ischemia-reperfusion injury. In our current work we have found that in activated macrophages, purines inhibit NO production, as assessed by the determination of nitrite, the stable end product of NO metabolism. This is in line with the PARP inhibitory effect of purines, as we and others have reported earlier that PARP inhibition reduces NO production by inhibition of iNOS mRNA expression (43, 53). Furthermore, we have previously published that fibroblasts derived from PARP<sup>-/-</sup> mice showed reduced expression of iNOS mRNA and protein, as compared to fibroblasts derived from wild-type mice (PARP<sup>+/+</sup>) (41). As the transcription factor NF $\kappa$ B is known to be a crucial regulator of iNOS gene expression and PARP has been shown to interact with NF $\kappa$ B and other transcription factors as well (42, 64), inhibition by purines of NO production may be the consequence of an interference by PARP inhibition of NF $\kappa$ B activation. Thus, we hypothesize that, in addition to cytoprotection (i.e., direct protection against oxidant induced necrosis by PARP inhibition), some of the antiinflammatory effect of purines may also be explained by the reduced production of inflammatory mediators such as NO.

### Regulation of the mode of cell death (apoptosis vs. necrosis by hypoxanthine)

The role of PARP in mediating the cytotoxic effect of peroxynitrite is conveniently characterized in thymocytes, a frequently used model system for cell death. Using a multiparametric approach, we have previously shown that 1) low concentrations of peroxynitrite causes apoptotic cell death (caspase activation, DNA fragmentation); 2) high concentrations of peroxynitrite cause necrosis (plasma membrane disruption, mitochondrial alterations, inhibition of apoptotic parameters); and 3) activation of PARP and consequent ATP depletion divert the default apoptotic death pathway toward necrosis (48, 51). The same pattern of modulation of cell death is seen with agents that indirectly interfere with PARP activation, such as cellular calcium chelators or zinc chelators (49, 50). In our current work we have shown that hypoxanthine, in line with its PARP inhibitory properties, dose-dependently inhibited the peroxynitrite and SIN-1-induced necrosis of thymocytes. Similar to PARP inhibitors such as 3-aminobenzamide, nicotinamide, or 5-iodo-6-amino 1,2-benzopy-

rone, hypoxanthine diverted peroxynitrite-induced necrosis toward apoptosis, as indicated by increased caspase activation and DNA fragmentation. This effect of PARP inhibitors can be explained by the preservation of cellular ATP required for the energy-demanding apoptotic process. We have also shown before that oxidant-induced and PARP-mediated rapid necrotic cell death is associated with mitochondrial alterations such as mitochondrial membrane depolarization and secondary superoxide production (51), similar to perturbations characterizing prolonged apoptotic cell death. Hypoxanthine also inhibited peroxynitrite-induced and PARP-mediated mitochondrial alterations, providing further support for PARP being the primary target of hypoxanthine in its cytoprotective effect. Thus, taken together, purines appear to work, at least in the currently used model systems, as nonprofessional but reasonably potent inhibitors of PARP activation; they modulate cell death in a fashion that is entirely consistent with their PARP inhibitory effect.

The current results are the first to demonstrate that an endogenous molecule (other than nicotinamide) inhibits PARP activation and therefore modulates oxidant-induced cell death in a physiologically or pathophysiologically relevant concentration range. We propose the hypothesis that endogenously produced purines (such as hypoxanthine) inhibit PARP activation in various forms of reperfusion injury and inflammation, and thereby modulate the fate of the oxidant-exposed parenchymal cells. FJ

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