

# Nucleotide Depletion Due to Reactive Oxygen Metabolites in Endothelial Cells: Effects of Antioxidants and 3-Aminobenzamide

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**ABSTRACT.** Reactive oxygen metabolites have an important role in ischemia-reperfusion injury. One of the sources of reactive oxygen metabolites is xanthine oxidase, which is present in several tissues but is also released into the circulation after ischemia. We studied the effect of several potentially protective compounds on adenine nucleotide depletion induced by extracellular xanthine oxidase and hypoxanthine, in concentrations relevant to human pathophysiology. In umbilical vein endothelial cells prelabeled with  $^{14}\text{C}$ -adenine, cellular adenine nucleotides retained  $64 \pm 9\%$  of the initial radioactivity over a 4-h incubation with culture medium (controls), whereas in the presence of xanthine oxidase (80 mU/mL) and hypoxanthine (100  $\mu\text{M}$ ), only  $3 \pm 4\%$  of radioactivity remained in cellular nucleotides, the rest appearing in catabolic products in the medium. Glutathione and 3-aminobenzamide, an inhibitor of poly-ADP-ribose polymerase, partly prevented the nucleotide depletion (adenine nucleotide radioactivity  $15 \pm 6\%$  to  $33 \pm 13\%$  of total), but scavengers of the hydroxyl radical, dimethylthiourea and DMSO, as well as vitamins E and C, were without effect. Superoxide dismutase prevented the leakage of nucleotides into the culture medium but not intracellular nucleotide catabolism, whereas the latter process was decreased by catalase, consistent with predominant effects of superoxide and hydrogen peroxide at the cell membrane and interior, respectively. (*Pediatr Res* 34: 572-576, 1993)

## Abbreviations

3-ABA, 3-aminobenzamide  
CAT, catalase  
DMTU, dimethylthiourea  
GSH, glutathione  
ROM, reactive oxygen metabolites  
SOD, superoxide dismutase  
XO, xanthine oxidase  
LDH, lactate dehydrogenase

ROM have been ascribed a significant role in ischemia-reperfusion injury (1). Free radicals can cause enzyme inactivation and DNA strand breaks, as well as protein and lipid damage leading to alterations in membrane structures (2). An important source of these radicals is the enzyme XO (EC 1.1.3.22), which is generated in ischemic tissue from its physiologically occurring form, xanthine dehydrogenase. At the same time, the substrate

of XO, hypoxanthine, accumulates and upon reperfusion is converted into xanthine and uric acid, with reactive oxygen species as by-products (3-5). In the neonatal period, and especially in preterm infants, a number of important clinical problems have been ascribed to ROM (6, 7).

In man, XO activity has been found mainly in the liver, kidney, and intestine (8). XO may be released from perfused rat liver during and after ischemia (9), and significant XO activity was found in plasma after release of tourniquet-induced ischemia in patients undergoing surgery of the upper extremity (10). Another extracellular source of free radicals is granulocytes (11), which accumulate in postischemic tissue. Although many cell types, e.g. epithelial cells, fibroblasts, and hepatocytes, may be damaged by ROM (12), the endothelial cell is a prime target as shown by microscopic studies in several models of ischemia-reperfusion injury (13, 14). Also, granulocytes attach primarily to endothelial cells in tissues after ischemia.

We have previously characterized the effects of extracellular XO and hypoxanthine on human umbilical vein endothelial cells, using depletion of radioactive adenine nucleotides from prelabeled cells as a sensitive index of cell damage. In this study, we tested several compounds previously shown to prevent or modify ROM-induced cell damage with respect to their ability to prevent nucleotide depletion induced by XO and hypoxanthine. The concentrations of the effectors were in the range encountered in clinical situations, and those of the protective compounds were in the range achievable *in vivo*.

## MATERIALS AND METHODS

**Cell culture and labeling.** Endothelial cells were cultured from human umbilical veins as described previously (15) and used before the 6th passage. Briefly, the cells were detached with 0.2% collagenase (Worthington Biochemical Corporation, Freehold, NJ) and grown to confluency in 25-cm<sup>2</sup> culture flasks (Nunc, Roskilde, Denmark) coated with 0.2% gelatin (Merck, Darmstadt, Germany) in F-10 medium (Gibco Europe, Paisley, UK) containing 20% pooled sterilized human serum (Finnish Red Cross, Helsinki, Finland) at 37°C (5% CO<sub>2</sub>-95% air). For the experiments, cells were transferred to gelatin-coated wells with a diameter of 1.5 cm (Multidish, Nunc) and cultured in F-10 medium containing 20% human serum until confluent. The cells were then prelabeled with  $^{14}\text{C}$ -adenine (sp act 51-55 mCi/mmol; Amersham International, Amersham, UK), final concentration 0.1 mM. After 16-18 h, the labeled medium was removed and the cells were washed three times with purine-free RPMI 1640 medium (Gibco Europe).

**Incubation with test compounds.** For actual experiments, serum-free RPMI 1640 medium was added to each multidish well, followed by either 5, 10, or 15 mM GSH (Merck); 300 or 600 IU/mL (220  $\mu\text{g}/\text{mL}$ ) SOD (Cu,Zn-SOD, EC 1.15.1.1, Sigma Chemical Company, St. Louis, MO); 300 or 600 IU/mL (60  $\mu\text{g}/\text{mL}$ ) CAT (Sigma, EC 1.11.1.6); SOD and CAT; 5, 10, or 15

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mM DMTU (Aldrich-Chemie, Steinheim, Germany); 5 or 10 mM DMSO (Merck); 50 or 100  $\mu$ M  $\alpha$ -tocopherol (Sigma); 100  $\mu$ M or 10 mM ascorbic acid (Merck); or 5, 10, or 15 mM 3-ABA (Sigma). Then, after 15 min of preincubation, 100  $\mu$ M unlabeled hypoxanthine (Sigma) and 80 mU/mL XO (Boehringer Mannheim GmbH, Penzberg, Germany) were added. These concentrations were previously found to result in significant depletion of cellular adenine nucleotides (15), which was considered a suitable basis for assessing the effects of scavengers. The commercial XO preparation has protease contamination, but we have previously shown that the cellular nucleotide depletion is not due to the protease activity (15). In some experiments, we also used a protease-free commercial XO preparation (Biozyme Laboratories Ltd., Blaenavon, UK). After incubation at 37°C (5% CO<sub>2</sub>, 95% air) for 4 h, the medium was removed and frozen, and the cells were cooled on ice, extracted twice with 100  $\mu$ L of ice-cold 0.4 M perchloric acid, and frozen at -20°C until analysis.

**Analysis of purine compounds.** Nucleotides in the cell extracts and medium and their catabolic products (hypoxanthine, xanthine, uric acid) in the medium were separated and their radioactivities measured by thin-layer chromatography as previously described (15).

**<sup>51</sup>Cr-release from endothelial cells.** To obtain an independent assessment of free radical damage, cells were incubated overnight with 1  $\mu$ Ci/mL <sup>51</sup>Cr (sodium chromate, Amersham International), washed three times, and incubated for 4 h with XO and hypoxanthine in the presence and absence of 5 mM GSH. Release of <sup>51</sup>Cr was calculated in terms of cpm released from the test cells into the medium as percent of the maximum released from cells treated with 0.4 M perchloric acid.

**Expression of results and statistical methods.** <sup>14</sup>C-adenine labeled mainly the adenine nucleotide pool; other purine nucleotides contained 3  $\pm$  1% and nucleic acids 11  $\pm$  5% of the radioactivity taken up by the cells ( $n$  = 8). Because the latter were unaltered in the course of the experiments, the results are expressed as the percent distribution of radioactivity between adenine nucleotides (cells and medium separately) and their catabolic products (hypoxanthine, xanthine, uric acid). The interassay variation was 5% in thin-layer chromatography. Previous work with different types of cultured cells has shown that there is no difference between the metabolism of <sup>14</sup>C-labeled and unlabeled nucleotides (16).

Experiments were performed in duplicate at least twice. BMDP statistical software was used for data analysis (17). Comparisons between groups were done using the nonparametric Mann-Whitney U test and  $p$  < 0.05 was considered significant. Results are expressed as means  $\pm$  SD.

## RESULTS

**Nucleotide depletion by XO and hypoxanthine.** In the presence of 100  $\mu$ M hypoxanthine, 80 mU/mL XO caused a profound nucleotide depletion; in 4 h, cellular adenine nucleotides decreased to 3  $\pm$  4% (control 64  $\pm$  9%) and catabolic products in the medium increased to 71  $\pm$  6%; over 95% of the catabolic products were uric acid and xanthine (control 32  $\pm$  7%, over 90% was hypoxanthine) (Fig. 1). After control incubations, an average of 5  $\pm$  4% (Fig. 1) of the total radioactivity were in nucleotides in the medium. Treatment with hypoxanthine and XO increased this to 23  $\pm$  10% (Fig. 1). However, because of significant ectonucleotidase activity on endothelial cells (18), part of the nucleotides released may appear as catabolic products under the present experimental conditions.

**GSH.** GSH alone had no effect on endothelial cell adenine nucleotides (data not shown). In incubations with XO and hypoxanthine, there was a dose-dependent but only partial prevention of cellular nucleotide depletion (Fig. 1). Concomitantly, there was a decrease in the radioactivity in the catabolic products but no significant change in nucleotides in the medium.

To test the effects of GSH with another assay, release of <sup>51</sup>Cr

from prelabeled cells was measured. XO and hypoxanthine together increased the <sup>51</sup>Cr release up to 71  $\pm$  14% (spontaneous release in the control cells, 19  $\pm$  6%), and incubation with both XO and hypoxanthine together with 5 mM GSH decreased the release to the level of 50  $\pm$  11% (Fig. 2). Thus, exposure to the superoxide-generating system and the cytoprotective effect of GSH are reflected similarly in our nucleotide depletion assay and chromium release.

**Antioxidant enzymes.** SOD alone had no effect on cellular adenine nucleotides, but it prevented the leakage of nucleotides into the medium induced by XO and hypoxanthine. Intracellular nucleotides decreased to 11  $\pm$  1% of the total radioactivity taken up by the cells compared with 10  $\pm$  1% in the absence of SOD (Fig. 3), but nucleotides in the medium decreased to 7  $\pm$  1% compared with 11  $\pm$  2% in the absence of SOD. CAT alone had a marked protective effect on the depletion of cellular nucleotides, which remained at the level of 38  $\pm$  3% of total radioactivity, and a matching decrease in catabolic products was observed (Fig. 3). However, CAT had no significant effect on the leakage of nucleotides into the medium (Fig. 3). When both SOD and CAT were present in the medium together with both XO and hypoxanthine, no additive protective effect was seen (Fig. 3), inasmuch as the decreased nucleotide leakage and decreased intracellular catabolism were in the range observed with either enzyme alone.

Smaller amounts of these enzymes, that is 300 IU/mL, had no effect on the nucleotide depletion due to the combination of XO and hypoxanthine (data not shown).

**Free radical scavengers.** Neither 5 or 10 mM DMSO nor 5 to 15 mM DMTU decreased the loss of adenine nucleotides caused by XO and hypoxanthine or altered the leakage of nucleotides or catabolic products into the medium (data not shown).

**Antioxidant vitamins.** Neither  $\alpha$ -tocopherol nor ascorbic acid modified the adenine nucleotide depletion induced by hypoxanthine and XO (data not shown). To explore the long-term effects of  $\alpha$ -tocopherol, cells were cultured in the presence of 50 or 100  $\mu$ M of the vitamin for 3 d. No protective effect was obtained, inasmuch as after the exposure to hypoxanthine and XO unsupplemented cells contained 15  $\pm$  2% of total radioactivity in adenine nucleotides ( $n$  = 4) compared with 14  $\pm$  1% in cells supplemented with 50  $\mu$ M and 12  $\pm$  2% in cells supplemented with 100  $\mu$ M  $\alpha$ -tocopherol. The distribution of radioactivity between the catabolic products and nucleotides in the medium was also unaltered.

**3-ABA.** When an inhibitor of poly-ADP-ribose polymerase, 3-ABA, was present in the culture well with XO and hypoxanthine, partial protection from nucleotide depletion was seen (Fig. 4). The effect was almost maximal at the lowest concentration studied.

## DISCUSSION

The main by-product of the hypoxanthine/XO reaction is the superoxide anion, but some hydrogen peroxide is also generated. The superoxide radical is converted into the hydrogen peroxide by a dismutation reaction, and if ferrous iron is present, into the hydroxyl radical in an iron-catalyzed Fenton reaction (19). Evidence bearing on the role of these compounds in cell injury has usually been indirect, based on the effect of antioxidant enzymes or free radical scavengers in preventing damage. The results have been quite variable, depending on the experimental system and the method of assessing damage.

In our model, adenine nucleotide depletion may be due to intracellular catabolism and/or membrane damage, not necessarily lethal, resulting in leakage of intact nucleotides out of the cytoplasm. The exact contribution of these two mechanisms is difficult to estimate, because endothelial cells have active ectonucleotidases (18), which would catabolize extruded nucleotides and lead to underestimation of membrane leakage. Our previous findings, obtained by using an inhibitor of ecto-5'-nucleotidase,

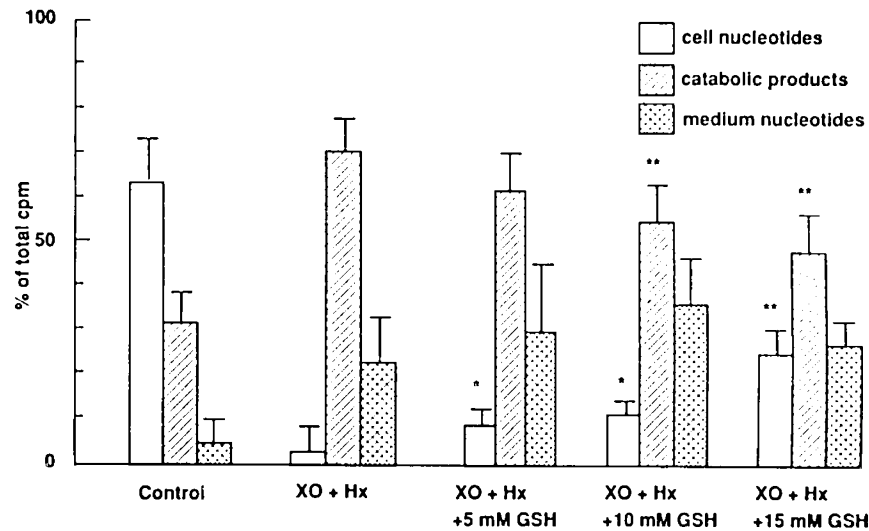


Fig. 1. Prevention of adenine nucleotide depletion by GSH. Endothelial cells were pre-labeled with  $^{14}\text{C}$ -adenine, incubated 4 h with 80 mU/mL XO and 100  $\mu\text{M}$  hypoxanthine (Hx) (except control), with or without 5 or 15 mM GSH. Results are expressed as % of total soluble radioactivity in cells and medium. *Open columns*, cellular adenine nucleotides; *hatched columns*, sum of catabolic products (hypoxanthine, xanthine, and uric acid) in the medium; *crosshatched columns*, sum of nucleotides in the medium. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with XO + hypoxanthine group. Mann-Whitney U test,  $n = 4$ .

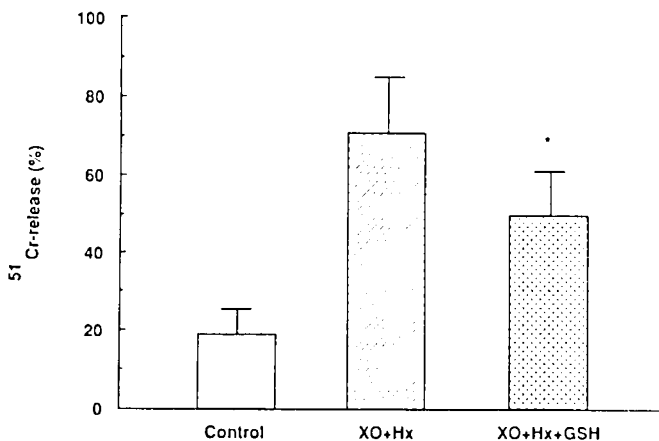


Fig. 2.  $^{51}\text{Cr}$ -release from endothelial cells and prevention by GSH. Cells pre-labeled with  $^{51}\text{Cr}$ , incubated with 80 mU/mL XO and 100  $\mu\text{M}$  hypoxanthine (Hx) (except control cells) with or without GSH (5 mM). *Open column*, control; *hatched column*, XO and hypoxanthine; *cross-hatched column*, XO, hypoxanthine, and GSH. \*,  $p < 0.05$  (Mann-Whitney U test,  $n = 4$ ).

indicate that a superoxide-generating system (XO + hypoxanthine), but not hydrogen peroxide, causes membrane damage and leakage of nucleotides (20). We have also shown that depletion of pre-labeled nucleotides corresponds very closely to measurements of total nucleotide levels by liquid chromatography (20). This rules out significant resynthesis of ATP during exposure to ROM.

XO activity in endothelial cells is unmeasurably low (15). Thus, our model reflects the effects of free radicals generated extracellularly by XO in the circulation (10) or by granulocytes (11). We have also shown that exposure to the superoxide-generating system decreases ecto-5'-nucleotidase activity of endothelial cells (21). This influences the pattern of metabolites in the extracellular fluid but not nucleotide depletion itself.

The concentration of XO used in this study is similar to levels reported locally *in vivo* (9, 10, 22). We have studied the dose response to XO (without hypoxanthine) and found that 1 mU/mL of XO causes a significant decrease in nucleotide levels in endothelial cells (15). Endogenous production of hypoxanthine

was sufficient for a modest effect, but nucleotide depletion was increased by addition of exogenous hypoxanthine at concentrations encountered in clinical situations (23, 24).

GSH is known as an important component of the cellular defenses against free radical damage. Depletion of GSH aggravates XO-xanthine-induced epithelial cell death and membrane damage, as assessed by the release of LDH from cells (25), and at concentrations from 2 to 5 mM extracellular GSH protects endothelial cells from ATP depletion caused by hydrogen peroxide (26). We observed only partial but dose-dependent prevention of nucleotide depletion by GSH and very little effect on the leakage of nucleotides into the culture medium. GSH is poorly transported into endothelial cells (26, 27), and it may act as a scavenger of free radicals (2, 25). Thus, it is possible that the effects of glutathione in our experiments were extracellular. Protection of the ischemic kidney by *i.v.* GSH may be another example of extracellular effects of this compound (28).

In cultured fibroblasts and endothelial cells exposed to XO and acetaldehyde or hypoxanthine, CAT at concentrations from 250 to 1000 U/mL has been found more effective in preventing cell death, as assessed by release of  $^{51}\text{Cr}$ , than SOD at concentrations from 75 to 500 U/mL (12, 29). In our experiments, both enzymes had only modest effect, which was entirely consistent with previous data. We have shown that XO plus hypoxanthine, but not hydrogen peroxide, causes a leakage of nucleotides from endothelial cells, whereas the latter compound induces catabolism of intracellular nucleotides (20). Accordingly, SOD modified nucleotide leakage, whereas CAT markedly decreased intracellular catabolism (Fig. 3). Failure of SOD to prevent catabolism may be caused by continued formation of hydrogen peroxide by dismutation and partly by XO itself.

In studies with trypan blue uptake or release of  $^{51}\text{Cr}$  from pre-labeled cells as the end point, CAT has been more protective, relatively speaking, than in our experiments (5, 12). This may be because nucleotide depletion is a more sensitive, but not necessarily irreversible, index of free radical effects (30). In this study, concentrations of the antioxidant enzymes SOD and CAT lower than 300 mU/mL did not protect the cell from injury due to ROM. Excessive concentrations, higher than 600 mU/mL, could prevent damage because of the protective effect of any big protein molecule, similarly to albumin (31).

DMSO and DMTU are scavengers mainly of the hydroxyl radical (12, 32). They failed to prevent nucleotide depletion in

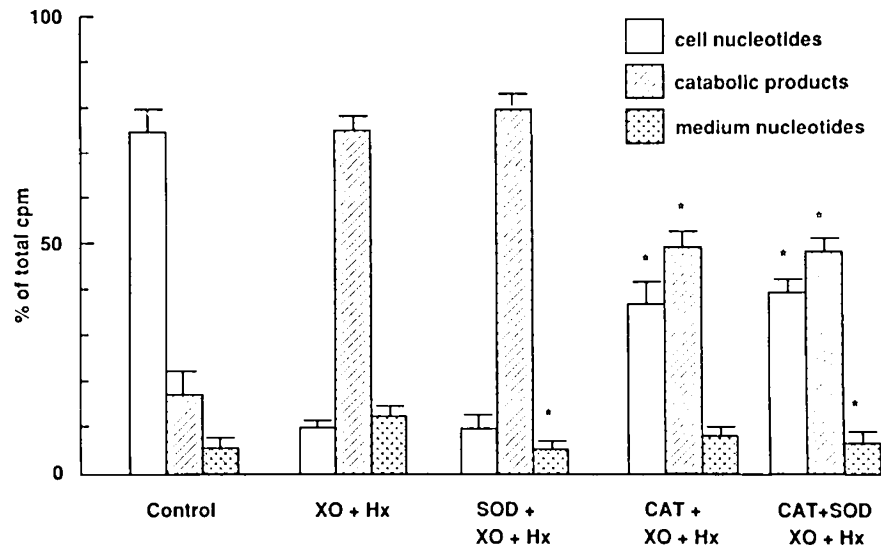


Fig. 3. Prevention of adenine nucleotide leakage to the medium with SOD, or prevention of nucleotide depletion by CAT and the combination of SOD and CAT. Prevention of nucleotide depletion or leakage from endothelial cells prelabeled with  $^{14}\text{C}$ -adenine for 4 h with or without 80 mU/mL XO and 100  $\mu\text{M}$  hypoxanthine (Hx) with or without 600 IU/mL SOD or CAT as indicated. Expression of results and symbols are as in Figure 1.

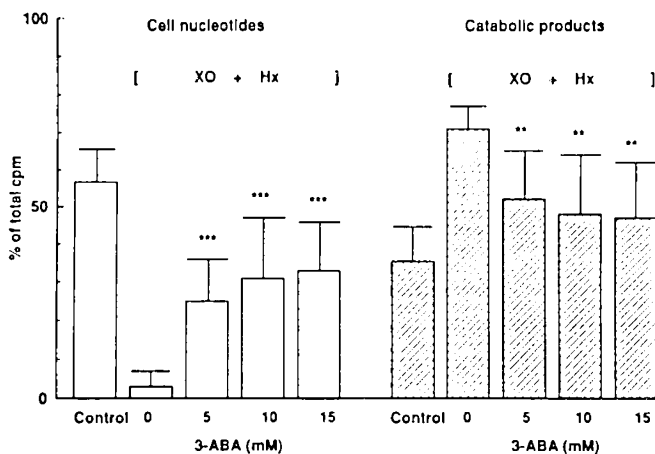


Fig. 4. Prevention of nucleotide depletion with 3-ABA. Prelabeled cells (with  $^{14}\text{C}$ -adenine) were incubated with 80 mU/mL XO and 100  $\mu\text{M}$  hypoxanthine (Hx) as indicated (except control) with or without different concentrations of 3-ABA as indicated. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  compared with XO + hypoxanthine group (Mann-Whitney U test,  $n = 4$ ).

our model at levels previously found to protect endothelial cells from injury induced by XO + hypoxanthine or hydrogen peroxide as assessed by  $^{51}\text{Cr}$  or LDH release (12, 32). DMSO and DMTU protected mice against oxygen toxicity but they did not inhibit trichloroacetic acid induced lipid peroxidation *in vitro* at levels similar to those we have used (33). This suggests either that nucleotide depletion and endothelial cell death as assessed by  $^{51}\text{Cr}$  or LDH release do not have a simple causal relationship or that the hydroxyl radical does not play a significant role under our experimental conditions. The endothelial cells used in our experiments were beyond the second passage. Their iron content may be lower than in uncultured endothelial cells (34). This implies lower production of the hydroxyl radical, which may explain the lack of effect of DMSO and DMTU. In other studies, DMTU has prevented cell death due to hydrogen peroxide (12, 35). The discrepancy with our results may again depend on the different criterion for assessment.

$\alpha$ -Tocopherol exerts its antioxidant effect in biologic membranes (2, 34). Because it had no protective effect when present in the incubation medium together with XO and hypoxanthine,

we attempted to "load" cell membranes by prolonged incubation with  $\alpha$ -tocopherol. However, nucleotide depletion was unaffected by this treatment. The explanation may be that under ordinary culture conditions there is sufficient antioxidant present for maximal effect (36), which cannot be augmented by an excess. We have previously demonstrated the protective effect of serum on ROM-induced injury (15), which was mainly attributable to albumin. The remaining approximate 20% of the effect was not characterized, but could be due to vitamin C or E or uric acid (37). The cells in that study, as well as in the present experiments, were grown in F-10 medium containing 20% human serum before the actual exposures. They may thus have been provided with ample antioxidant vitamins. We did not study the possible role of uric acid present in serum.

The enzyme poly-ADP-ribose polymerase is activated by DNA damage, promoting DNA repair but also depletion of NAD and ATP (38). Its inhibitor, 3-ABA, at a concentration of 2.5 mM, has been found to prevent ATP depletion (39) but was less effective in our experiments. Again, the problem may be the site of action. If membrane leakage contributes significantly to adenine nucleotide depletion, as indicated by our recent findings (20), an intracellularly acting compound, such as 3-ABA, could not have a major protective effect.

Most of the agents studied in the present experiments have been found more effective in preventing ROM-induced cell death when either unreasonably high concentrations or less sensitive indicators of irreversible damage have been used (5, 12, 25). It seems that endothelial cells tolerate severe nucleotide depletion without necessarily losing their viability. A similar conclusion was reached in studies in which inhibition of energy metabolism was used to induce nucleotide depletion in endothelial cells (40). Functional disturbances preceding irreversible damage have previously been shown upon exposure of endothelial cells to hydrogen peroxide (41). Such disturbances, like nucleotide depletion used in our studies, may be too sensitive to provide optimal parameters for assessing cytoprotection. Nevertheless, a thorough *in vitro* evaluation is essential before the clinical use of potential antioxidant compounds is contemplated.

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