In vivo inosine protects alveolar epithelial type 2 cells against hyperoxia-induced DNA damage through MAP kinase signaling

S. Buckley, L. Barsky, K. Weinberg, and D. Warburton

1Developmental Biology and 2Research Immunology/Bone Marrow Transplant Programs, Saban Research Institute, Children’s Hospital of Los Angeles, Los Angeles, California

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Buckley, S., L. Barsky, K. Weinberg, and D. Warburton. In vivo inosine protects alveolar epithelial type 2 cells against hyperoxia-induced DNA damage through MAP kinase signaling. Am J Physiol Lung Cell Mol Physiol 288: L569–L575, 2005. First published December 3, 2004; doi:10.1152/ajplung.00278.2004.—Inosine, a naturally occurring purine found in certain foods such as meat and sugar beets, is formed by the breakdown of adenosine by adenosine deaminase and has anti-inflammatory properties (17). Inosine can reduce LPS-induced acute lung injury: mice treated with inosine 1 h after tracheal instillation of LPS showed reduced lung proinflammatory cytokines and improved lung morphology when compared with untreated animals (22). Herein, we examine the potential of inosine treatment as a modulator of subacute hyperoxic lung injury to the AEC2, using our well-established adult rodent model. We show that in vivo inosine is effective in reducing hyperoxic damage to the AEC2. The metabolite of inosine, uric acid, when administered in vitro to AEC2 from hyperoxic rats, completely blocks hyperoxia-induced DNA damage. These data suggest that inosine and its metabolites deserve further evaluation for their potential to reduce specific hyperoxic damage to the pulmonary alveolar epithelium.

METHODS

Oxygen and in vivo inosine treatment. Animal experiments were approved by our Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats were exposed to short-term hyperoxia as described previously (6). Briefly, rats were placed in a 90% oxygen/10% nitrogen mixture for 4 h. Rats were then placed in an endotoxin-free PBS at the start of hyperoxic treatment and every 12 h thereafter for 48 h. This dose has been shown to ameliorate LPS-induced lung injury (22), a more extreme damage model than sublethal hyperoxia. Control animals were injected with PBS. At the end of the exposure period the rats were anesthetized by inhalation of isoflurane. After complete exsanguination by cannulation of the left ventricle, animals were perfused with 0.15 M phosphate buffered saline (PBS) to remove blood. The lungs were explanted and snap frozen in liquid nitrogen.

8-Oxoguanine DNA glycosylase (OGG1) is a base excision repair enzyme that repair 8-oxoG damage in DNA. This enzyme is expressed in lung tissue and is activated in response to hyperoxia (8). The inosine treatment group demonstrated a significant decrease in OGG1 activity when compared to the control group. This suggests that inosine treatment may provide protection to the lung by enhancing DNA repair and reducing the formation of 8-oxoguanine, a mutagenic lesion that can cause DNA damage and cell death.

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Address for reprint requests and other correspondence: D. Warburton, MS 35, Children’s Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027 (E-mail: dwarburton@chla.usc.edu).

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Saline perfusion via the pulmonary artery, the lungs were lavaged to remove macrophages. The lavaged lungs were then used for AEC isolation and culture.

Bronchoalveolar lavage. Lung lavage fluid was obtained as part of the AEC isolation procedure. The lungs were perfused in situ with PBS at 37°C through the pulmonary artery until blood-free. The lungs and trachea were then removed en bloc to a sterile dish. The lungs were gently lavaged to capacity eight times with fresh volumes of isotonic salt solution before the instillation of elastase (11). The bronchoalveolar lavage (BAL) was pooled on ice and centrifuged to pellet cells. Lavage fluid containing red blood cells was discarded. The cell-free supernatant was stored in aliquots at −70°C. The cells were counted and were consistently >90% macrophages.

Isolation and culture of adult AEC. AEC2 were isolated from lavaged lungs by elastase digestion, followed by differential adherence on IgG plates as described by Dobbs et al. (11). The cells were cultured in HEPES-buffered DMEM with 10% FBS on Primaria tissue culture plates for 24 h to achieve maximal attachment. Immuno-staining of attached cells after 24-h culture with a surfactant protein C (SP-C) antibody confirmed that ≥95% of the attached cells were positive for SP-C. In experiments requiring uncultured cells, the AEC2 were used immediately after perfusing the IgG plates.

In vitro inosine treatment. Inosine was added to the AEC cultures from the time of plating at a dose of 1 mM. This dose was determined by titration of inosine on hyperoxic cultures, using 0.1, 1, and 10 mM doses and measuring DNA damage [terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL)]. Inosine at 0.1 M had virtually no effect, whereas 1 and 10 mM had similar effects (data not shown).

TUNEL labeling. DNA strand breaks were measured in 1% paraformaldehyde-fixed AEC2 by labeling with FITC-dUTP, using an APO-DIRECT kit from PharMingen (San Diego, CA) according to the manufacturer’s instructions. FITC-labeled cells counterstained with propidium iodide (PI) were analyzed with a Becton Dickinson FACScan and CellQuest software, as previously described (4).

Cell cycle analysis. Cell cycle analysis was performed by FACS on cells that were PI-stained after 70% ethanol fixation. Becton Dickinson CellQuest and Verity ModFit LT 2.0 software were used for acquisition and analysis.

Glutathione measurement. Measurement of total glutathione (GSH plus GSSG) was performed on AEC2 after 24-h culture, as previously described (4). AEC2 were lysed in 1% Triton X-100 and were assayed immediately using a modification of the recycling method of Owens and Belcher (24). Glutathione levels were calculated relative to 1–4 nmol standards freshly prepared for each assay and corrected for protein.

Western blotting of proteins. Western analysis was performed on cell lysates as described by Bui et al. (7), using 20 μg of protein/lane. Equal loading was confirmed by blotting with an antibody to actin. Primary antibodies were directed against rat epitopes where possible. Proteins of interest were detected using horseradish peroxidase-linked secondary antibodies and the enhanced chemiluminescence system (Pierce) following the manufacturer’s instructions (Amersham, Arlington Heights, IL). Antibodies to phosphorylated (p)-ERK 1/2, ERK 2, p-p38, p-JNK, Bcl-2, poly(ADP-ribose) polymerase (PARP), SP-C, and interleukin-converting enzyme (ICE) were from Santa Cruz Biologics (Santa Cruz, CA). Antibody to human 8-oxoguanine DNA glycosylase (hOGG1) was from Novus Biologicals (Littleton, CO), antibodies to pSmad2 and Smad2 were from Cell Signaling Technology (Beverley, MA), and antibody to actin was from ICN (Irvine, CA). Secondary antibodies were from Sigma. We performed densitometric analysis of blots using Scion Image software from the National Institutes of Health, with blots from at least three independent experiments.

Transforming growth factor-β activity. Transforming growth factor (TGF)-β activity was measured in BAL using the plasmogen activator inhibitor (PAI-1) luciferase assay in mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to a firefly luciferase reporter gene (1). The cells were generously provided by Dr. Dan Rifkin of New York University. Addition of active TGF-β to these cells results in a dose-dependent expression of luciferase. Transfected mink lung epithelial cells were plated in 96-well plates, 1.6 × 10^4 cells/well in DMEM with 10% FCS, and allowed to attach for 3–4 h. The serum-containing medium was then removed, replaced with BAL diluted 1:1 with 2× DMEM + 0.2% BSA, and assayed in parallel with TGF-β standards in the same diluted medium. After 16–18 h of incubation, the cells were washed, lysed, and assayed for luciferase activity using the Enhanced Luciferase Assay Kit from PharMingen (San Diego, CA) and a Berthold luminometer (Zylux, Oak Ridge, TN).

Statistics. Data are presented as means ± SD. Statistical significance between treated and untreated groups was calculated using Student’s t-test, with P < 0.05 considered significant.

RESULTS

Inosine reduces DNA damage in AEC2 cultured from hyperoxic rats. In vivo hyperoxic damage to AEC2 DNA is detectable after 24-h culture, with increased TUNEL seen in AEC2 from hyperoxic animals compared with AEC2 from normoxic animals (5). To investigate the effect of inosine on hyperoxia-induced DNA damage, inosine was coadministered in vivo with 48-h hyperoxia treatment, as twice daily injections of 200 mg/kg ip, or added to AEC2 from hyperoxic animals at the time of plating (at a concentration of 1 mM) for 24-h culture. FACS analysis of TUNEL cultured AEC2 showed that inosine reduced hyperoxia-induced DNA damage by ~40% when used in vivo (Fig. 1). Interestingly, a similar reduction in DNA damage was seen with in vitro inosine, suggesting a direct effect on the AEC2.

Inosine, whether added in vivo to normoxic rats or in vitro to AEC2 from normoxic rats, had no effect on TUNEL, which was negligible, 5 ± 5%.

Inosine decreases the apoptotic profile of AEC2 cultured from hyperoxic animals. We have previously shown that AEC2 cultured from hyperoxic rats have a proapoptotic profile, with increased expression of the caspase ICE and decreased expression of the protective protein Bcl-2 (4). We examined the

Fig. 1. Hyperoxia-induced DNA damage was measured by fluorescence-activated cell sorting (FACS) analysis of FITC-dUTP labeled alveolar epithelial type 2 cells (AEC2) cultured from hyperoxic animals ± twice daily 200 mg/kg inosine in vivo or 1 mM inosine in vitro. Data are presented as means ± SD. Inosine reduced in vitro terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) of hyperoxic AEC2 (P < 0.05, n = 4) and in vivo TUNEL of hyperoxic AEC2 (P < 0.05, n = 7) compared with hyperoxia alone (n = 4). Inosine, whether administered in vivo (n = 3) or in vitro (n = 3), did not affect TUNEL of normoxic AEC, which was negligible, 5 ± 5%.
expression of ICE and Bcl-2, as well as the molecular nick sensor PARP, which is activated by oxidative stress (35), in lysates of AEC2 from hyperoxic animals ± in vivo inosine, using Western blotting. Inosine decreased the expression of ICE and the expression and degradation of PARP in AEC2 from hyperoxic rats, while increasing expression of Bcl-2 (Fig. 2).

Inosine reverses hyperoxia-induced glutathione depletion in AEC2 from hyperoxic rats. We and others have shown that the DNA damage seen in cultured AEC2 from hyperoxic animals correlates with depleted cellular GSH (4, 33). In contrast, AEC2 cultured from hyperoxic, inosine-treated animals had significantly higher GSH (approximately twofold) than cells from hyperoxic animals without inosine treatment (Fig. 3). Inosine administered in vitro to AEC2 from hyperoxic animals was equally effective in increasing hyperoxia-depleted glutathione levels, suggesting that inosine directly affects the antioxidant status of AEC2. Inosine, either in vivo or in vitro, had no significant effect on GSH levels in AEC2 from normoxic rats.

Inosine reduces the proportion of hyperoxic AEC2 in S and G2/M phases of the cell cycle. To examine the effect of inosine on the AEC2 cell cycle, we analyzed PI-labeled cells by FACS. AEC2 have an essentially quiescent phenotype, with the majority of cells in G0/G1 and only a small proportion (~6%) in S and G2/M phases, which increases transiently to ~10% during the repair phase after hyperoxia (7). When cultures of AEC2 from control and hyperoxic animals were incubated for 24 h, ~1 mM inosine, fixed in 70% ethanol, and stained with PI for FACS analysis, the inosine-treated AEC2 had significantly fewer cells in S and G2/M phases than untreated cells. Inosine administered in vivo during the hyperoxic exposure also resulted in an accumulation of cells in G0/G1 in AEC2 cultured from these animals, whereas inosine administered in vivo to normoxic rats had no effect (Fig. 4).

Inosine increases the levels of active TGF-β in BAL. In our model of acute hyperoxia, active TGF-β, derived from alveolar macrophages, is recovered from the BAL from hyperoxic animals, whereas there is negligible active TGF-β in the BAL of normoxic animals (6). However, BAL levels of active TGF-β recovered from inosine-treated hyperoxic rats were consistently threefold higher than the levels from simply hyperoxic animals (Fig. 5). It may appear counterintuitive that protection against hyperoxia is associated with an exaggerated TGF-β hyperoxic response, rather than a downmodulation. However, increased active TGF-β in the alveolar milieu may contribute to the G1 cell cycle block seen in AEC2 isolated from inosine-treated hyperoxic animals, thereby allowing more time for DNA repair. In contrast to TGF-β, levels of the...
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In vivo, inosine is effective in reducing hyperoxia-induced DNA damage. We have previously shown that MEK inhibitor PD 98059 abolishes the protective effect of in vivo inosine on hyperoxia-induced DNA damage. We have previously shown that MEK inhibitor PD 98059, when added at 50 μM to AEC2 cultures from the time of plating, causes sustained inhibition of ERK1/2 activity, which persists for at least 24 h (4). Because in vivo inosine results in ERK activation that is sustained in culture, we determined the effect of in vitro MEK blockade on inosine-mediated protection. When AEC2 from hyperoxic animals were treated with 50 μM PD 98059, added from the time of plating for 24-h culture, the protective effect of inosine was abolished, resulting in TUNEL similar to that seen in AEC2 from rats subjected to hyperoxia alone (Fig. 8). This suggests that the protective effect of inosine is associated with MAP kinase signaling, downstream of MEK, and that this signaling is sustained during culture.

In vivo inosine increases ERK activation in AEC2. The AEC2-specific DNA damage seen in our hyperoxia model can be reduced by culture on biological substrates that approximate the alveolar basement membrane, conditions that increase ERK activation (4). In vivo inosine administered with hyperoxia resulted in a significant twofold activation of ERK1/2 over and above the activation seen in hyperoxic cultured AEC2, as measured by Western blotting using an antibody specific to the active, phosphorylated form of the enzyme (Fig. 7). In vivo inosine also increased ERK activation in AEC2 from control normoxic animals, but not to a significant level. Inosine-induced ERK activation was not associated with altered activation of other MAP kinase family members, JNK or p38, and inosine added in vitro did not activate ERK (data not shown).

MEK inhibition using PD 98059 abolishes the protective effect of in vivo inosine on hyperoxia-induced DNA damage. We have previously shown that MEK inhibitor PD 98059, when added at 50 μM to AEC2 cultures from the time of plating, causes sustained inhibition of ERK1/2 activity, which persists for at least 24 h (4). Because in vivo inosine results in ERK activation that is sustained in culture, we determined the effect of in vitro MEK blockade on inosine-mediated protection. When AEC2 from hyperoxic animals were treated with 50 μM PD 98059, added from the time of plating for 24-h culture, the protective effect of inosine was abolished, resulting in TUNEL similar to that seen in AEC2 from rats subjected to hyperoxia alone (Fig. 8). This suggests that the protective effect of inosine is associated with MAP kinase signaling, downstream of MEK, and that this signaling is sustained during culture.

In vivo inosine induces expression of the DNA repair protein OGG1. To examine the role of inosine in promoting DNA repair after hyperoxic injury, we chose OGG, a base excision repair enzyme, as a marker (15). OGG is activated by hyperoxia (37). Western blotting using an antibody directed against human OGG (hOGG1) showed virtually undetectable OGG expression in control AEC2 and induction with hyperoxia.

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The metabolite of inosine, uric acid, is also effective in reducing hyperoxia-induced DNA damage and restoring glutathione levels. Inosine has a relatively short half-life in the bloodstream. A major metabolite of inosine is uric acid (29), which is a potent antioxidant. To investigate whether uric acid is as effective as inosine in reducing hyperoxia-induced DNA damage, we added uric acid at the same concentration used for inosine, 1 mM, to control and hyperoxic AEC2 at the time of

Fig. 6. Representative Western blot shows that AEC2 from control rats have barely detectable phosphorylated (p)-Smad2 levels, as detected with an antibody directed against the active, phosphorylated form of the enzyme. Inosine coadministered with hyperoxia increases Smad2 activation over and above hyperoxic levels by 1.9 ± 0.6 fold, as measured by scanning densitometry (n = 4).

Fig. 7. Lysates of AEC2 cultured 24 h from control and hyperoxic animals were analyzed by Western blotting for ERK1/2 activation, using an antibody directed against the active, phosphorylated form of ERK1/2 (pERK). This figure is representative of several blots and shows that ERK1/2 activity in cultured AEC2 is increased by in vivo hyperoxia per se and also increased a further 2-fold over hyperoxic levels by in vivo inosine administered to hyperoxic rats (P < 0.05, n = 3). The nonphosphorylated form of ERK was used as a loading control. No changes were detected in the activities of JNK or p38, as measured by blotting with antibodies to p-JNK and p-p38 (data not shown).
plating and cultured the cells for 24 h. Uric acid, added in vitro to AEC2 cultured from hyperoxic animals reduced TUNEL to control levels (Fig. 10A) and also completely restored hyperoxia-depleted GSH levels (Fig. 10B). TUNEL of normoxic AEC2 is negligible and did not change with uric acid treatment.

DISCUSSION

Protection of the pulmonary alveolar epithelium during oxygen therapy would be key to the prevention of oxygen-mediated human diseases, such as bronchopulmonary dysplasia (BPD) in the premature neonate. Understanding and manipulating the balance between hyperoxic damage and repair in the epithelium necessitate the use of rodent models, despite differences between human and rodent (19). Exposure of the rodent alveolar epithelium to high levels of oxygen results in ablation of AEC1 and damage to the AEC2 (30), the proposed alveolar epithelial progenitor cell population. In our model of sublethal hyperoxia, the AEC2 that survive hyperoxia have depleted glutathione levels, increased DNA damage, and increased expression of apoptotic markers when cultured compared with AEC2 from normoxic animals (5). Herein we show that, in contrast, AEC2 isolated and cultured from hyperoxic animals treated with inosine as adjunct therapy show significantly less damage. Our data suggest that inosine is working both directly, and indirectly through paracrine effects, on the AEC2.

Inosine is anti-inflammatory when administered to the lung (22). However, we did not detect a significant decrease in the levels of hyperoxia-induced proinflammatory cytokines TNF-α and IL-1β in the BAL, as measured by rat-specific ELISAs (data not shown). In contrast, active levels of TGF-β recovered from the BAL were threefold higher in the inosine treated hyperoxic animals compared with animals treated with hyperoxia alone. We speculate that increased active TGF-β in the lung milieu may contribute to the accumulation of AEC2 in G0/G1, which may provide a time-out for DNA repair. Protection against oxygen-induced DNA damage through G1 blockade has been reported in mink lung epithelial cells and is attributed to an enhanced ability to limit or rapidly recognize
ERK activation plays a protective role in maintaining mitochondrial function and regulating caspase activity under conditions of cellular stress (21), and ERK activation in AEC2 in vitro can protect the cell from hyperoxia-induced DNA damage (4). Herein, we show that the protection against specific hyperoxic damage to AEC2 conferred by inosine is coincident with MAPK signaling that is initiated in the animal, since it is apparent in fresh isolates as well as cultured AEC2 from inosine-treated hyperoxic animals. Because PD 98059, an MEK inhibitor, abolishes the protective effect of in vivo inosine on hyperoxia-mediated DNA damage to AEC2, it appears that inosine works through upstream signaling pathways converging on MEK. Because in vitro inosine does not activate ERK in hyperoxic AEC2, ERK activation resulting from in vivo inosine may be a result of paracrine secretion of cytokines and/or growth factors within the lung that are protective to the AEC2. It has recently been reported that TGF-β cytokines and/or growth factors within the lung that are pro-inflammatory when cultured from hyperoxic rats. Using Western blotting with a commercially available antibody generated against rat A3R, molecular mass ~52 kDa, we could not detect a protein of the expected molecular mass in AEC2 lysates, although it was readily detectable in lysates of whole rat lung. However, a major band of ~30 kDa was seen in AEC2 lysates, as well as in our positive control, consisting of macrophage lysate protein (data not shown). Whether this is a metabolite of the receptor or an unrelated protein remains to be determined, and heterogeneity of A3Rs has been reported (10). Inosine can also bind and activate rodent adenosine A2a receptors (14). We could not detect the presence of A2a receptors on AEC2 using available antibodies, with the caveat that these antibodies were not generated against rat epitopes (data not shown). Confirmation and identification of adenosine AR subtypes on AEC2 using PCR will be the focus of future studies. If adenosine receptors are indeed present, in vivo blocking experiments using receptor-specific antagonists can then be designed to determine whether inosine is protective to the AEC2 through direct AR signaling.

Uric acid, the major metabolite of inosine, and itself an antioxidant, is as effective as inosine in reducing hyperoxia-induced DNA damage to the AEC2. Inosine, whether injected or ingested, causes only a minor increase in serum inosine levels in rats, with a more sustained elevation in serum uric acid levels (29). Thus when prophylactic inosine is administered as a bolus, as in our model of sublethal hyperoxic lung injury and in other rodent models using a similar mode of delivery, such as LPS-induced lung injury (22), and stroke (9), it is likely that uric acid contributes to the protective effect of inosine.

We conclude from this initial study that in vivo inosine treatment, given as a twice daily boluses of 200 mg/kg is useful as an adjunct to short-term hyperoxic therapy in the adult rodent and protects the AEC2 from specific forms of hyperoxic damage. It has a direct protective effect on AEC2, possibly though the antioxidant effect of its metabolite, and also an indirect effect, mediated through other cell types, when administered in vivo. We speculate that a continuous infusion of inosine, administered by osmotic pump or intravenously, may afford better protection than twice daily intraperitoneal injections. Given the important caveat that humans may respond differently from rodents, our data suggest that hyperoxic damage to the alveolar epithelium could be minimized using an in vivo treatment that is readily available, cost effective, and well tested in humans at doses exceeding those used in our experiments (23). Reducing hyperoxic damage to the neonatal lung could be an important potential application of inosine or metabolites as adjunct therapy to hyperoxia, and studies are currently are under way in our laboratory. Indeed, uric acid levels in the tracheobronchial aspirates of preterm babies correlate with FiO₂ during the first 3 days of life and are much higher than levels reported in adults, suggesting that uric acid may be physiologically important as an antioxidant in the respiratory tract during the first days of life (34).

We therefore speculate that inosine and its metabolites are also worth evaluating for reducing hyperoxic lung damage in a neonatal setting, with the ultimate goal of ameliorating or preventing BPD.

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