OZONE INHIBITS ENDOTHELIAL CELL CYCLOOXYGENASE ACTIVITY THROUGH FORMATION OF HYDROGEN PEROXIDE

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ABSTRACT

We have previously demonstrated that a 2H exposure of cultured pulmonary endothelial cells to ozone (0.0-1.0 ppm) in-vitro resulted in a concentration-dependent reduction of endothelial prostacyclin production (90% decrease at the 1.0 ppm level). Ozone-exposed endothelial cells, incubated with 20 uM arachidonate, also demonstrated a significant inhibition of prostacyclin synthesis. To further examine the mechanisms of the inhibition of prostacyclin synthesis, bovine pulmonary endothelial cells were exposed to 1.0 ppm ozone for 2H. A significant decrease in prostacyclin synthesis was found within 5 min of exposure (77 $\pm$ 36% of air-exposed control values, $p < 0.05$). Endothelial prostacyclin synthesis returned to baseline levels by 12H after ozone exposure, a time point which was similar to the recovery time of unexposed endothelium treated with 0.5 uM acetylsalicylic acid. Incubation of endothelial cells, previously exposed to 1.0 ppm ozone for 2 hours, with 4 uM PGH_2 resulted in restoration of essentially normal prostacyclin synthesis. When endothelial cells were co-incubated with catalase (5U/ml) during ozone exposure, no inhibition of prostacyclin synthesis was observed. Co-incubation with either heat-inactivated catalase or superoxide dismutase (10U/ml) did not affect the ozone-induced inhibition of prostacyclin synthesis. These data suggest that H_2O_2 is a major toxic species produced in endothelial cells during ozone exposure and responsible for the inhibition of endothelial cyclooxygenase activity.

INTRODUCTION

Ozone, derived through a complex chemical process in which sun energy interacts with oxides of nitrogen and hydrocarbons in the lower atmosphere, is the principal oxidant air pollutant present in photochemical smog (1). The lung is a major site of eicosanoid metabolism derived from the selective oxidation of arachidonic acid (2). It has been suggested that oxygenation products of arachidonic acid may be important and critical mediators in the development of ozone-induced lung injury (3).

Although a well-recognized effect of ozone is pulmonary edema formation, the exact cellular mechanism(s) of ozone-induced lung vascular injury is not well understood (4). Pulmonary vascular endothelial cells are the most abundant cell type in human lungs, comprising 20-30% of the cell population (5), and synthesize the major portion of prostacyclin in the lung (2,6). Pulmonary endothelial cells are particularly sensitive to acute ozone damage. For example, inhalation of 1.0 ppm ozone for 1-2 days in mice results in morphologic alterations in the pulmonary circulation including swelling and lysis of endothelial cells (7). We have previously demonstrated that a 2 hour exposure of cultured pulmonary endothelial cells to ozone (0.0-1.0 ppm) in-vitro resulted in a significant concentration-dependent reduction of endothelial prostacyclin and thromboxane production. Exposure to the highest ozone concentration studied, 1.0 ppm, decreased prostacyclin production by 90%. This occurred in spite of the fact that ozone caused an increased release of arachidonic acid from cellular phospholipid pools (8). When the ozone-exposed endothelial cells were incubated with exogenous arachidonic acid (20 uM for 5 min) the cells converted significantly less of this substrate into prostacyclin than did endothelial cells exposed to air. These results suggested that ozone exposure inhibited endothelial cyclooxygenase and/or prostacyclin synthetase activity.

The purpose of the present study was to determine in cultured pulmonary endothelial cells exposed in-vitro to ozone: the site of ozone-induced enzyme inhibition (i.e., cyclooxygenase and/or prostacyclin synthetase); the time course for inhibition and recovery of endothelial prostacyclin production; and the nature of the toxic species produced during ozone exposure responsible for the observed inhibition of endothelial prostacyclin synthesis.

GENERAL METHODS

Cell Culture

Pulmonary artery endothelial cells were harvested by gentle scraping of the intimal surface of the main stem pulmonary arteries of calves (9). The cells were dispersed into 25 cm^2 culture flasks (Corning Glass Works, Corning, NY) containing Medium 199 supplemented with 10% fetal calf serum (FCS), penicillin (100 ug/ml), gentamicin (50 ug/ml), streptomycin (100 ug/ml), thymidine (5 ug/ml) and deoxycytidine (5 ug/ml), grown to confluence in 5% CO_2, 95% air at 37°C as previously described (8).

Ozone Exposure System

Endothelial cells were grown to confluence in 125 ml volume glass roller bottles (Bellco Glass, Inc., Vineland, NJ) in media supplemented with 10% FCS and 50 mM HEPES.

Prior to exposure, the cells were washed thoroughly with serum-free media and 8.0 ml of serum-free Medium 199 with 50 mM HEPES added to the bottles. The roller bottles with confluent cells were placed on a roller apparatus housed within an enclosure that was maintained at 37°C. Ozone was continuously generated by ultraviolet irradiation of ultrapure air (Spectronics Corporation, Westbury NY). The ozonated air was introduced into the bottles at a constant flow of 10 ml/min and the ozone concentration was continuously monitored by chemiluminescence at the bottle inlet and maintained within ±3% of the desired level (Model 350-2R, Meloy Laboratories, Springfield, MA). The exposure system has been previously described (8).

Radioimmunoassay for Arachidonate Metabolites

After exposure of the cells to ozone, the media was removed, centrifuged for 15 min at 2000 x g, and the supernatant was frozen in liquid nitrogen and stored at -70°C until analysis. Aliquots of the supernatant were used for duplicate measurements of 6-keto-$PGF_{1\alpha}$ by radioimmunoassay (New England Nuclear Corp., Boston, MA). The cells were removed by trypsin after exposure and counted by hemocytometry.

For experiments involving the addition of exogenous agents following ozone exposures, cells were washed (x 2) with serum-free medium immediately after the 2H exposure period, 8.0 ml of serum-free medium added and the cells incubated with exogenous agents: arachidonic acid (20 uM for 5 min; dissolved in ethanol to give a 0.6% final concentration of alcohol in the media; 99% purity, porcine origin, Sigma Chemical Co., St. Louis, MO), or PGH_2 (4 uM for 2 min; dissolved in ethanol for a final alcohol concentration of 0.6%; >85% purity, Oxford Biomedical Research, Inc., Oxford, MI).

Assay of thiobarbituric acid-reaction products

Lipid peroxidation was assessed in exposed endothelial cells by measurement of thiobarbituric acid-reactive (TBA) products using a spectrophotometric method (10). Following exposure to air or ozone, the cells were removed from the bottles by treatment with trypsin for 10 min, centrifuged at 2000 x g for 10 min and the cell pellet resuspended in 25 ml of phosphate buffered saline (PBS) for assay of TBA-reactive products. An aliquot (1.0 ml) of sample was combined with 1.0 ml of trichloroacetic acid (TCA) (10% TCA in 0.3 M HCl, w/v). The solution was centrifuged (1500 x g, 15 min), 1.0 ml of the supernatant was added to 200 ul of TBA solution (0.12 M TBA in 0.26 M Tris), $FeSO_4$ added (final concentration of 2.5 mM), and butylatedhydroxytoluene (BHT) added (final concentration of 0.25 mM). The mixture was then heated for 15 min at 95°C. Absorbance was measured at 532 nm.

Ram Seminal Vesicle Preparation

Ram seminal vesicle microsomes were prepared as described previously (11). All studies were carried out in 66 mM Tris buffer (pH 7.8) with 1 mM DETAPAC, in a final volume of 1.5 ml. Microsomal protein content was 19.5 mg/ml. Cyclooxygenase activity was measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). After a 1 min incubation at 37°C, exogenous H_2O_2 in 20 ul Tris buffer (final concentration of 100 uM) was added. Following 1 min of incubation with H_2O_2, exogenous arachidonic acid in ethanol was added to a final concentration of 100 uM. This incubation procedure ensured that all exogenous peroxide-dependent cyclooxygenase activity would be complete prior to addition of arachidonic acid.

Measurement of Hydrogen Peroxide

Hydrogen peroxide was assayed by monitoring absorbance in phenol red at 610 nm (12). A 200 ul aliquot of serum-free media was added to 1.0 ml of a phenol red and horseradish peroxidase solution [10 ul of a horseradish peroxidase solution (66.7 mg in 10 ml PBS with glucose) and 10 ul phenol red (10 g/1.0 ml PBS with glucose without Ca^{++} or Mg^{++})]. The reaction mixture was incubated at 37°C for 1H before the absorbance was measured. The detectability limit of H_2O_2 was approximately 1 uM.

Materials

Medium 199, trypsin, and PBS were obtained from the UNC Lineberger Cancer Research Center (Chapel Hill, NC). Fetal calf serum was purchased from Hyclone Laboratories (Logan, UT) and gentamicin sulfate obtained from Valley Biologicals (State College, PA) or Lymphomed, Inc. (Melrose Park, IL). Protein assay kits were purchased from Bio-Rad (Richmond, CA). All other chemicals were reagent grade and obtained from Sigma Chemical Co., St. Louis, MO.

Statistics

Data were analyzed using the student's t-test for paired or unpaired variates (13). Group differences among several different treatments were compared by a one-way analysis of variance or a Scheffe' test. A value of $p < 0.05$ was considered statistically significant. All data were reported as mean $\pm$ SEM.

RESULTS

We have previously demonstrated that addition of exogenous substrate (20 uM arachidonate for 5 min) to pulmonary endothelial cells, previously exposed to 1.0 ppm ozone for 2H, resulted in a reduced synthesis of prostacyclin (64% decrease in prostacyclin production at the 1.0 ppm level compared to air-exposed controls). These findings suggested that ozone exposure resulted in an

inhibition of endothelial cyclooxygenase and/or prostacyclin synthetase enzymatic activities. To further examine this, confluent pulmonary endothelial monolayers were exposed to ozone in an identical fashion. Following a 2H exposure to 1.0 ppm ozone, the media was removed, the cells washed extensively and then incubated with PGH_2 (4 uM for 2 min). The results are shown in Table 1. In preliminary experiments, this concentration of PGH_2 and duration of incubation with normal unexposed pulmonary endothelial cells resulted in a 32-fold increase in prostacyclin production by 2 min, compared to basal synthesis, and the response was linear up to 3 min after addition of the cyclic endoperoxide.

Table 1
EFFECTS OF INCUBATION OF PGH_2 WITH PULMONARY ENDOTHELIUM AFTER OZONE EXPOSURE[a]

O_3 dose	n	6-keto-$PGF_{1\alpha}$ (ng/10^6 cells) post-PGH_2 incubation	p[b]
0.0 ppm	7	14.7 ± 5.0	-
1.0 ppm	7	12.1 ± 1.5	NS

[a]Endothelial cells were exposed to 1.0 ppm O_3 or air for 2H as described in the text. The cultures were then washed and immediately incubated with 4 uM PGH_2 for 2 min. The amount of prostacyclin (as 6-keto-$PGF_{1\alpha}$) produced by the cells into the media after PGH_2 incubation was measured. The data are expressed as mean ± SEM.
[b] Significant difference from corresponding 0.0 ppm value.

This concentration (1.0 ppm) and duration of ozone exposure (2H) resulted in a 90 ± 10% inhibition in prostacyclin synthesis (data not shown). Following incubation with the endoperoxide, PGH_2, the amount of prostacyclin produced into the media by the ozone-exposed endothelial cells was not significantly different from the amount synthesized by the control, air-exposed endothelial cells (82 ± 10% of controls, p=NS). These results suggest that endothelial cyclooxygenase and not prostacyclin synthetase activity was inhibited by ozone exposure.

The time course of inhibition of prostacyclin synthesis was determined during a 2H exposure to either 1.0 ppm ozone or air by serially removing aliquots (0.5 ml) of media from the bottles during the exposure. The data are shown in Figure 1. Endothelial cells exposed to air for two hours produced a constant amount of prostacyclin during the 2H exposure period (average

production of 290 ± 15 pg/10^6 cells for all time points). Cells exposed to 1.0 ppm ozone showed a decreased synthesis of prostacyclin by 5 min after the start of the exposure (23 ± 11% of air-exposure values) with maintenance of this depressed rate of synthesis throughout the remaining exposure period (average production of 98 ± 9 pg/10^6 cells for all time points). The production rate by the air-exposed cells was significantly greater than that of the ozone-exposed cells at every time point studied ($p < 0.05$). These data suggest that the decrease in endothelial synthesis of prostacyclin occurred rapidly upon exposure to ozone. Furthermore, the ozone-induced decrease in prostacyclin synthesis was not due to an early initial increase in prostacyclin synthesis resulting in self deactivation of cyclooxygenase.

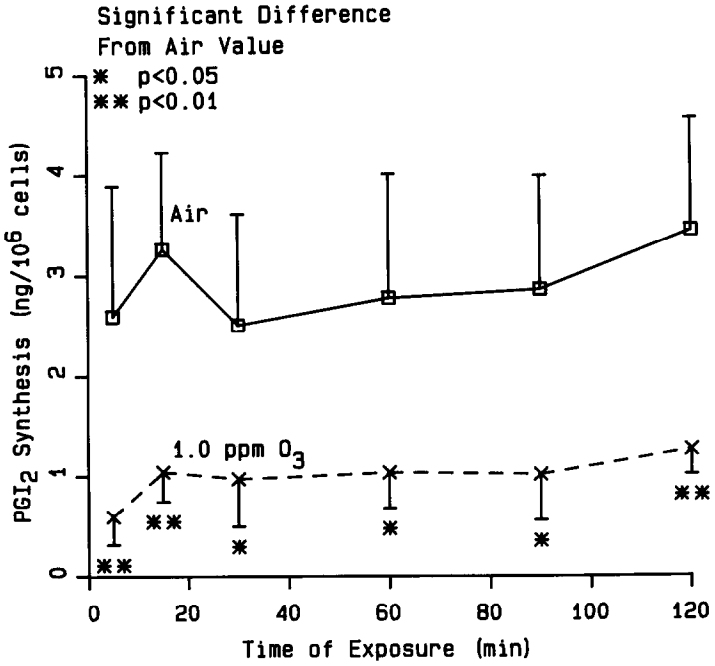

Figure 1. The amount of prostacyclin produced by pulmonary endothelial cells during a 2H exposure to either 1.0 ppm ozone or air. The data are shown as ng metabolite/10^6 cells (mean ± SEM) for 4-6 experiments.

In additional experiments, some cell cultures (n=5) were exposed to ozone, washed extensively, incubated with arachidonic acid (20 uM for 5 min) and the media analyzed

for 6-keto-$PGF_{1\alpha}$ (time zero). The remaining cultures were washed twice, 8.0 ml of serum-free media supplemented with 50 mM HEPES and the cultures were then incubated at 37°C for 12H or 24H. After the termination of the incubation, the media was removed, the cells were incubated with arachidonic acid (20 uM for 5 min) in serum-free media and the media were analyzed for 6-keto-$PGF_{1\alpha}$. The results are shown in Figure 2.

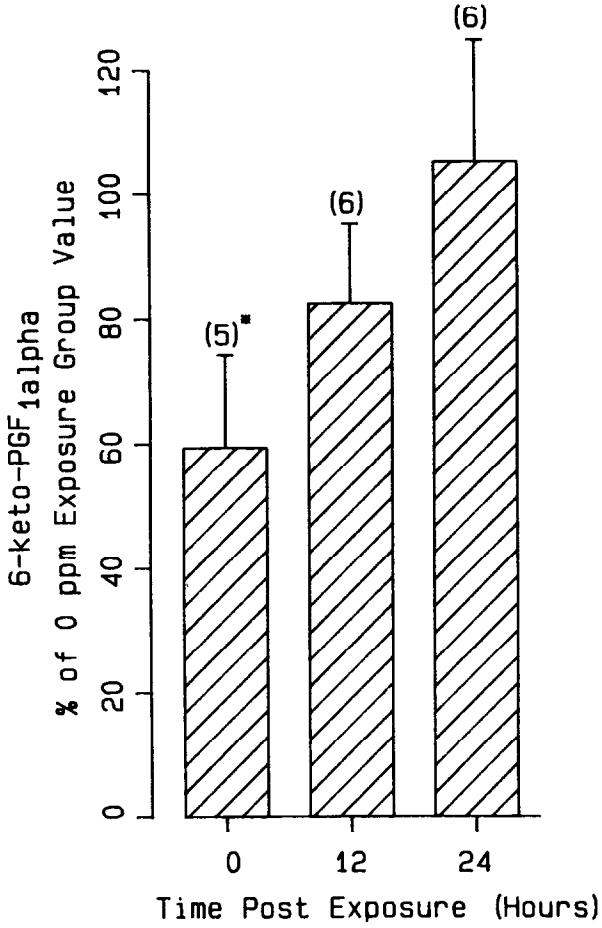

Figure 2. Time course of recovery of endothelial prostacyclin synthesis in cells previously exposed to 1.0 ppm ozone for 2H. The data are shown relative to the simultaneously obtained, control air-exposure values (mean ± SEM). * Significant difference ($p < 0.05$) from controls.

Endothelial cells grown to confluence, exposed to 1.0 ppm ozone for 2H and then incubated with 20 uM arachidonate for 5 min, had a significant decrease in prostacyclin synthesis of 42 ± 10% compared with control cultures immediately after the 2H exposure period (time zero, $p < 0.05$). The ozone-exposed endothelial cells recovered 83 ± 13% (compared to air-exposed control cultures, p=NS) of their ability to synthesize prostacyclin by 12H after the end of the ozone exposure period. By 24H, the ozone-exposed endothelial cell production of prostacyclin was 105 ± 20% of control cultures (p=NS). This time course of recovery of prostacyclin synthetic ability was similar to the time for recovery found in the same endothelial cell lines incubated with a known irreversible inhibitor of cyclooxygenase, acetylsalicylic acid (ASA). We used an ASA concentration of 0.5 uM which, in preliminary studies, resulted in a similar degree of enzymatic inhibition after a 2H incubation to that observed after a 2 hour exposure to 1.0 ppm ozone (60% versus 41% inhibition respectively, n=5, p=NS). By 12H after ASA incubation, endothelial cells treated with ASA produced similar (92 ± 14%, n=6) amounts of prostacyclin compared with cultures treated with vehicle alone (0.05% ethanol in serum-free media v/v, p=NS) following incubation with 20 uM arachidonic acid for 5 min. No further significant increase in prostacyclin production was found 24H after ASA treatment. The cells remained viable (>90% excluded trypan blue) and attached to the bottles (>99% of the cell population remained adherent) at 24H post air or ozone exposure and ASA or vehicle incubation.

It has been suggested that ozone may produce its toxic effects through the formation of oxygen free radicals and peroxides. The following studies were performed to examine the role of toxic oxygen radicals and lipid peroxides in the mechanism of ozone-induced inhibition of endothelial prostacyclin synthesis. Specifically, the effects of the free radical scavengers, catalase and superoxide dismutase (SOD), in modulating the ozone-induced alteration in prostacyclin synthesis was examined. Additionally, we measured the concentrations of lipid peroxides in endothelial cell cultures exposed to either air or 1.0 ppm ozone. For these studies, endothelial cells were prepared for exposure as previously described. Prior to exposure, 8.0 ml of serum-free medium containing either catalase (5U/ml) or SOD (10 U/ml) was added to all bottles and the bottles rolled for 2 min at 37°C prior to exposure. In some studies, catalase was heated (56°C for 1 hr) in exposure medium immediately prior to addition into roller bottles in order to inactivate the enzymatic activity. The cultures were then

exposed to either 1.0 ppm ozone or air for 30 min. We chose this duration of ozone exposure since: 1) in preliminary experiments, we found no alteration of prostacyclin synthesis in unexposed endothelial cells incubated for 30 min with either catalase or SOD in the concentrations stated; and 2) maximal inhibition of prostacyclin synthesis during ozone exposure had occurred by this time point (Figure 1). Following exposure, aliquots of media were removed, centrifuged, and the supernatant analyzed for 6-keto-$PGF_{1\alpha}$. Values of the were expressed as per cent change compared to control air-exposure values. The data are shown in Figure 3.

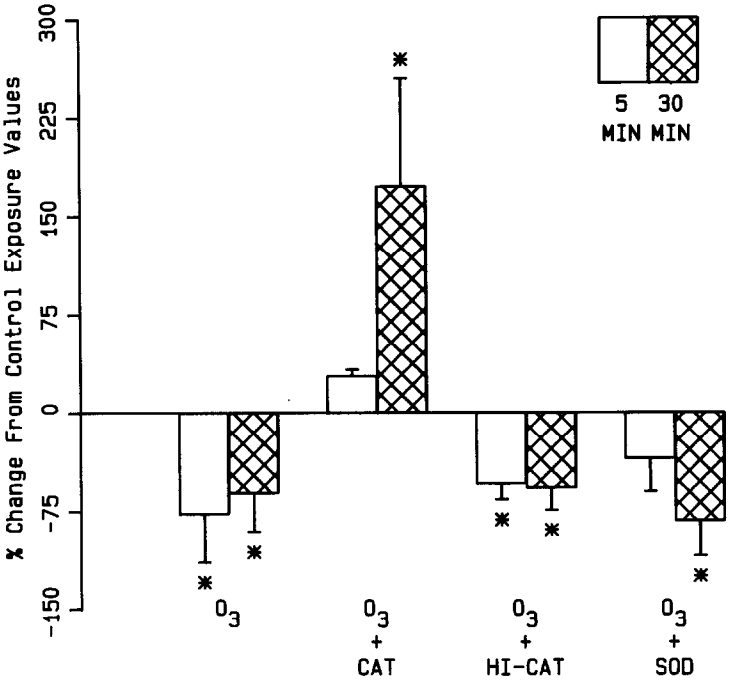

Figure 3: Effect of co-incubation with either catalase (CAT, 5U/ml, n=5), heat-inactivated catalase (HI-CAT, 5U/ml, n=5) or superoxide dismutase (SOD, 10U/ml, n=3) on endothelial prostacyclin synthesis during a 30 min exposure to 1.0 ppm O_3 or air. The amount of prostacyclin produced at 5 min (open bars) and 30 min (hatched bars), after initiation of ozone exposure, for each group are shown as the percent change from the air-exposed values at the identical time points (mean $\pm$ SEM). * Significant difference ($p < 0.05$) from control values.

Co-incubation of the endothelial cell cultures with either heat-inactivated catalase or SOD resulted in no alteration of the inhibition of prostacyclin production during the ozone exposure period (54% and 82% inhibition compared to air exposure at 30 min, respectively). In contrast, co-incubation of the endothelial cells with catalase during the exposure period protected against the ozone-induced inhibition of prostacyclin synthesis at 5 min (28% stimulation when catalase was present versus a 77% inhibition in the absence of catalase, $p < 0.01$). Interestingly, after 30 min of ozone exposure in the presence of catalase, there was an apparent stimulation of prostacyclin synthesis in the ozone-exposed endothelial cells. These data suggest that H_2O_2 plays a major role in the ozone-induced inhibition of endothelial cyclooxygenase activity.

Based on these results, suggesting that H_2O_2 is a toxic species generated by endothelial cells during exposure to ozone resulting in the marked inhibition of prostacyclin synthesis, we next determined if exogenous H_2O_2, directly added to the same endothelial cell lines, could also inhibit prostacyclin synthesis. Confluent endothelial cell cultures were exposed to H_2O_2 (50-100 uM) for 30 min, the media removed, fresh serum-free media added and the cells then incubated with 20 uM arachidonic acid for 5 min. At the end of each treatment period, medium was collected, centrifuged, and analyzed for 6-keto-$PGF_{1\alpha}$. Addition of H_2O_2 to endothelial cell cultures produced a decreased amount of prostacyclin [decreases of 32 $\pm$ 4% at 75 uM H_2O_2 (n=6) and 67% $\pm$ 53% at 100 uM H_2O_2 (n=6), compared to vehicle alone, $p<0.05$]. No decreases in prostacyclin production was seen at the 50 uM H_2O_2 dose. The cells remained viable (>95% excluded trypan blue dye) after 30 min of incubation with 100 uM H_2O_2. These data suggest that H_2O_2, in concentrations of 75-100 uM, inhibit endothelial prostacyclin production.

Hydrogen peroxide was not able to be detected in either the air-exposed or ozone-exposed (1.0 ppm) cultures at 5 and 120 min after the initiation of the ozone exposure. In order to ascertain whether endothelial cells may have rapidly decomposed H_2O_2 or generated an inhibitory substance preventing H_2O_2 detection, exogenous H_2O_2 (50 uM final concentration in 20 ml serum-free media) was added to a flask of unexposed confluent endothelial cells and incubated at 37°C. By 1 min after addition to the cells, no H_2O_2 was detectable. However, addition of the same 50 uM H_2O_2 solution to a flask containing media without cells resulted in a measured level of H_2O_2 of 44 uM after 5 min of incubation, which was similar to the added peroxide concentration of 50 uM. Thus, the inability to measure any H_2O_2 in the bathing media of ozone-exposed

cells was most likely due to increased decay of peroxide in the presence of cells or the production an inhibitory substance which interfered with the oxidation of phenol red used in the H_2O_2 detection assay.

In order to assess more directly the effects of H_2O_2 on cyclooxygenase activity, ram seminal vesicle microsomal preparations were directly exposed to 100 uM H_2O_2 (Figure 4). Addition of the peroxide resulted in a rapid (i.e., within 1 min) inhibition of cyclooxygenase activity (Figure 4B).

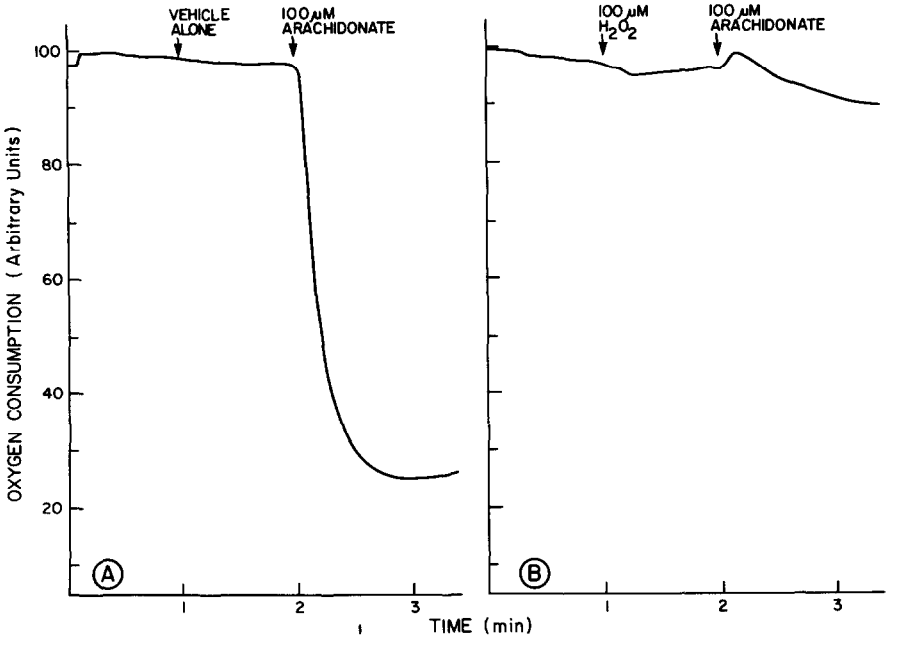

Figure 4: Effect of addition of either the vehicle alone (4A) or 100 uM H_2O_2 (4B) on cyclooxygenase activity in ram seminal vesicle microsomal preparations. See text for more complete details.

Assessment of lipid peroxidation by the measurement of TBA-reactive products (Figure 5) revealed differences in the absorbance of homogenates of air-exposed cells compared to the ozone-exposed cells (1.0 ppm for 2H). These data indicate that TBA-reactive products, presumably lipid peroxides, were found in both the air and ozone-exposed cultures after a 2H exposure. Air-exposed cells had a constant absorbance throughout the TBA reaction period. In contrast, endothelial cells exposed to ozone (1.0 ppm for 2H) contained greater amounts of thiobarbituric acid-reactive products than cells exposed to air alone. The increased absorbance in the ozone-exposed cells occurred at 5 and 10 min of reaction with TBA.

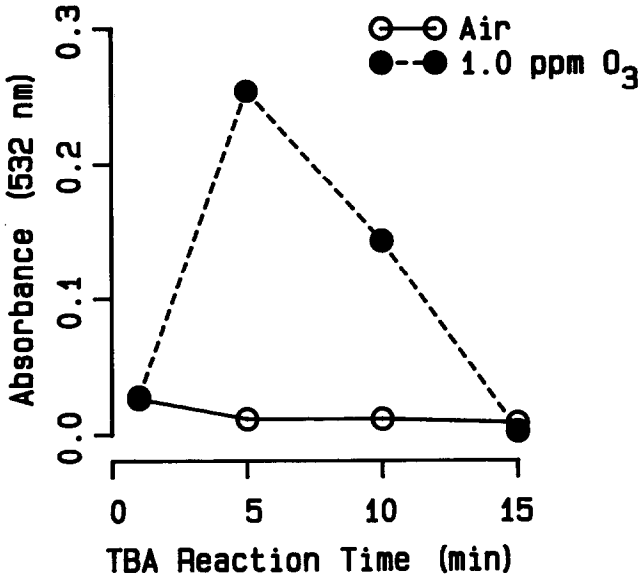

Figure 5: Production of thiobarbituric acid-reactive products in pulmonary endothelial cells exposed to either 1.0 ppm ozone (dashed lines, closed circles) or air (solid lines, open circles) for 2H. See text for more complete details.

DISCUSSION

In summary, ozone exposure in-vitro results in a significant and rapid ($\leq$5 min) inhibition of endothelial prostacyclin synthesis (Figure 1). This decrease in prostacyclin production is not due to a decreased

availability of free arachidonic acid and when ozone-exposed cells were incubated with sufficient exogenous arachidonic acid, they produced less prostacyclin as compared to air-exposed controls (8). In contrast, incubation of ozone-exposed cells with the cyclic endoperoxide, PGH_2, essentially restored normal prostacyclin synthesis in ozone-exposed cells (Table 1). These data strongly suggest that the inhibited enzyme in the ozone-exposed pulmonary endothelial cells was cyclooxygenase. Cyclooxygenase has been reported as being bound both in the endoplasmic reticulum and the nuclear membrane (14) whereas prostacyclin synthetase is contained within the plasma membrane as well as the endoplasmic reticulum (15). Thus, the preferential effects of ozone on cyclooxygenase versus prostacyclin synthetase may be due to different subcellular locations of the two enzymes with the plasma membrane prostacyclin synthetase pool being more resistant to ozone-induced inhibition.

These in-vitro data are similar to results previously obtained in-vivo in dogs exposed to 1.0 ppm ozone for 1 hour (8). The in-vivo exposure to ozone resulted in an increase in pulmonary vascular resistance and alteration in pulmonary vascular reactivity to hypoxia similar to treatment of the dogs with a known cyclooxygenase inhibitor, indomethacin. Further support for the hypothesis that endothelial cyclooxygenase is inhibited by ozone is that the time course for recovery of prostacyclin synthesis in the ozone-exposed cells were similar to the recovery time after treatment with a known irreversible inhibitor of cyclooxygenase, ASA (Figure 2).

Ozone has been suggested to exert its toxicity through an oxidant related mechanism(s) (16). Based on the data presented in the present study, we suggest that H_2O_2, produced in endothelial cells during exposure to ozone, is a major toxic species responsible for the observed inhibition of endothelial cyclooxygenase activity. This hypothesis is supported by the experimental results that co-incubation of endothelial cells with catalase, during exposure to ozone, protected against the inhibition of cyclooxygenase (Figure 3). The mechanism for the protection afforded by catalase appeared to be enzymatic degradation of H_2O_2 since co-incubation of the cells with heat-inactivated catalase did not prevent a decrease in prostacyclin synthesis during exposure to ozone.

In separate experiments, the exogenous addition of 100 uM H_2O_2 to pulmonary endothelial cultures cells inhibited prostacyclin synthesis. Furthermore, when ram seminal vesicle microsomal preparations were directly exposed to a similar concentration of H_2O_2, a rapid decrease in cyclooxygenase activity of 90% was observed within 60 seconds after addition of the peroxide (Figure

4). The choice of using a H_2O_2 concentration of 100 uM was somewhat arbritary since we could not measure H_2O_2 directly in the exposed cells (see below). We chose the 100 uM H_2O_2 concentration based on our experimental results showing that concentrations of this magnitude inhibited arachidonic acid-stimulated prostacyclin production in intact pulmonary endothelial cells. A similar inhibitory effect of 100 uM H_2O_2 on cyclooxygenase activity was also demonstrated using ram seminal vesicle microsomal preparations (Figure 4). These data are very similar to the cited work of Whorton et al (23) who added known concentrations of H_2O_2 to cultured aortic endothelial cells. These investigators also demonstrated a rapid (within 1 min) inhibition of endothelial prostacyclin synthesis by H_2O_2, in the presence of exogenous arachidonic acid, and demonstrated a 90% inhibition of prostacyclin production at a H_2O_2 concentration of 100 uM (23). Harlan and Callahan, utilizing a glucose-glucose oxidase system to generate exogenous hydrogen peroxide in cultures of human umbilical vein endothelium, showed an increase in endothelial prostacyclin production in response to 100 mU glucose oxidase/ml (24). We do not have a specific explanation for the differences between this study and that of Whorton et al (23) or of our present studies. However, Harlan did not not measure the actual amount of H_2O_2 produced using glucose-glucose oxidase and therefore the amount of H_2O_2 actually produced in that study may have differed from the concentrations of exogenous H_2O_2 added in the present study or by Whorton et al (23). Also, these studies were performed without addition of exogenous arachidonate. The differing reported effects of H_2O_2 on "basal" and arachidonic acid-stimulated prostacyclin synthesis may be explained by possible differential sensitivities of cellular cyclooxygenase pools to peroxide. Willems et al (25) has reported the existence of multiple pools of cyclooxygenase in human endothelial cells.

Whorton et al (23) were able to detect exogenously added H_2O_2 incubated with aortic endothelial cells in the media 30 min after addition of 100 uM H_2O_2. The difference between this report and the data presented in the present study, demonstrating no detectable H_2O_2 within 1 min after addition to endothelial cultures, may be due to the cell type difference (pulmonary versus systemic), culture conditions, or method utilized for detection of this species. In the present study, the pulmonary endothelial cells may have rapidly degraded H_2O_2. This does not imply that H_2O_2 had not been formed by the ozone-exposed endothelial cells in the present study or that it was being degraded into a non-toxic species after ozone exposure. The data depicted in Figure 3, demonstrating

clear protection afforded by enzymatically-active catalase on ozone-induced endothelial cyclooxygenase inhibition, strongly suggests that H_2O_2 was formed by these cells during ozone exposure. In fact, the amount of H_2O_2 required to inhibit endothelial cyclooxygenase enzymatic activity need not have been very high. Markey et al (26) has demonstrated a rapid (<3 min) inhibition of cyclooxygenase, using purified ram seminal vesicle microsomal preparations, after incubation with H_2O_2 at concentrations of only 1-2 uM. Therefore, even if significant endothelial cell degradation of H_2O_2 occurred during ozone exposure in our experiments, there is a reasonable expectation that a high enough intracellular H_2O_2 concentration remained that would result in inhibition of cyclooxygenase activity.

These results, suggesting formation of H_2O_2 by ozone-exposed endothelium are consistent with the studies of Goldstein. This investigator, using an in-vivo rat ozone exposure model, demonstrated that inhalation of ozone resulted in the formation of H_2O_2 in erythrocytes. He suggested that H_2O_2, or a precursor, was being produced at the level of the pulmonary endothelium during inhalation of ozone (17). H_2O_2 has been shown to be able to diffuse across the plasma membrane (18,19). Endothelial cells have been shown to contain very low levels of endogenous catalase (20). In contrast, rat alveolar macrophages, a cell type containing high concentrations of endogenous catalase (21), has been recently shown to have an enhanced activity of cyclooxygenase in response to exposure to 1.0 ppm ozone for 2H in an identical fashion to the endothelial cells (22). These results in ozone-exposed macrophages are similar to the enhanced production of prostacyclin found 30 min after ozone exposure in the endothelial cells co-incubated with catalase (Figure 3).

It has been demonstrated that low concentrations of lipid hyodroperoxides are necessary to activate the biosynthesis of prostaglandins from arachidonic acid (27). Higher concentrations can inhibit cyclooxygenase. In the present study, endothelial cells exposed to ozone (1.0 ppm for 2H) contained greater amounts of TBA-reactive products than cells exposed to air exposure (Figure 5). This finding would support other studies which have shown that high lipid peroxide levels are produced during ozone exposure (28). The oxidation of lipids by H_2O_2 or a derivative could account for this increase in TBA-reactive products. Thus, lipid peroxides cannot be ruled out as an eventual toxic species resulting in the observed cyclooxygenase inhibition by ozone exposure. We would suggest that, based on our data demonstrating catalase-induced protection of cyclooxygenase inhibition

by ozone (Figure 3), that H_2O_2 appears to be the predominant toxic species involved in ozone-induced cyclooxygenase inhibition either via direct action on the cyclooxygenase enzyme or indirectly through formation of toxic derivatives such as lipid peroxidation products. Markey et al were able to demonstrate that the IC_{50} for H_2O_2 was 1.8 uM, resulting in cyclooxygenase inhibition; this concentration was comparable to the IC_{50} of several lipid peroxides suggesting that H_2O_2 can be as potent a direct inhibitor of cyclooxygenase as other toxic lipid species (26).

There are other possible endothelial cellular constituents, indirectly involved in the oxidation/reduction reactions converting arachidonate into prostacyclin, such as NADPH (which supplies reducing equivalents to regenerate reduced glutathione allowing continued glutathione peroxidase activity) which may also have been affected by ozone exposure. Decreased glutathione peroxidase activity following in-vivo ozone exposure have been reported (29). Other studies have demonstrated that short term (30 min) exposures to 2-3 ppm ozone inhibited glutathione reductase activity in rat lung homogenates (30) and oxidized NADPH (31). These ozone-induced effects could prevent the regeneration of both reduced glutathione and glutathione peroxidase activity. In contrast to these reports, chronic inhalation (7 days) of ozone by rats has been shown to cause an increase in lung glutathione peroxidase activity (32). Ozone-induced alterations in glutathione peroxidase activity does not seem as likely a possibility to have induced cyclooxygenase inhibition after ozone exposure because of the rapid time course of inhibition observed ($\leq$5 min). Inhibition of cyclooxygenase can also occur endogenously as a result of autooxidation of the enzyme due to the generation of an oxidant species (33). We do not believe this latter event is a possible mechanism since no early increase in prostacyclin production was ever noted during the ozone exposure period (Figure 1).

Further investigations into the molecular mechanisms of ozone-induced endothelial cyclooxygenase inhibition should help clarify the role of pulmonary arachidonate metabolism in the response of the lung to ozone.

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