

Effect of Hydrogen Peroxide on Prostaglandin Production and Cellular Integrity in Cultured Porcine Aortic Endothelial Cells

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Abstract

Oxidative damage to the vascular endothelium may play an important role in the pathogenesis of atherosclerosis and aging, and may account in part for reduced vascular prostacyclin (PGI₂) synthesis associated with both conditions. Using H₂O₂ to induce injury, we investigated the effects of oxidative damage on PGI₂ synthesis in cultured endothelial cells (EC). Preincubation of EC with H₂O₂ produced a dose-dependent inhibition (inhibitory concentration [IC₅₀] = 35 μ M) of PGI₂ formation from arachidonate. The maximum dose-related effect occurred within 1 min after exposure although appreciable H₂O₂ remained after 30 min (30% of original). In addition, H₂O₂ produced both a time- and dose-dependent injury leading to cell disruption, lactate dehydrogenase release, and ⁵¹Cr release from prelabeled cells. However, in dramatic contrast to H₂O₂ effects on PGI₂ synthesis, loss of cellular integrity required doses in excess of 0.5 mM and incubation times in excess of 1 h. The superoxide-generating system, xanthine plus xanthine oxidase, produced a similar inhibition of PGI₂ formation. Such inhibition was dependent on the generation of H₂O₂ but not superoxide in that catalase was completely protective whereas superoxide dismutase was not. H₂O₂ (50 μ M) also effectively inhibited basal and ionophore A23187 (0.5 μ M)-stimulated PGI₂ formation. However, H₂O₂ had no effect on phospholipase A₂ activity, because ionophore A23187-induced arachidonate release was unimpaired. To determine the effects on cyclooxygenase and PGI₂ synthase, prostaglandin products from cells prelabeled with [³H]arachidonate and stimulated with ionophore A23187, or products formed from exogenous arachidonate were examined. Inhibition of cyclooxygenase but not PGI₂ synthase was observed. Incubation of H₂O₂-treated cells with prostaglandin cyclic endoperoxide indicated no inhibition of PGI₂ synthase. Thus, in EC low doses of H₂O₂ potently inhibit cyclooxygenase after brief exposure whereas larger doses and prolonged exposure are required for classical cytolytic effects. Surprisingly, PGI₂ synthase, which is known to be extremely sensitive to a variety of lipid peroxides, is not inhibited by H₂O₂. Lipid solubility, enzyme location within the EC mem-

brane, or the local availability of reducing factors may explain these results, and may be important determinants of the response of EC to oxidative stress.

Introduction

The vascular endothelium is extremely sensitive to oxidative damage mediated by reactive oxygen metabolites either released from inflammatory cells (1-3) or produced within the endothelial cell (4). Of these metabolites hydrogen peroxide (H₂O₂) appears to be an important mediator of acute cellular injury in a variety of settings (2, 3, 5). Such oxidative damage may play a role in the pathogenesis of atherosclerosis (6) and is postulated to contribute to the aging process (7, 8). Traditionally, oxidative damage in cultured endothelial cells has been assessed by markers such as ⁵¹Cr release, lactate dehydrogenase (LDH)¹ release, or by cell detachment from culture plates (2, 3, 9). These markers of damage are crude in that they are generally indicative of severe cell membrane disruption and cell death. More subtle, but none the less important, biochemical changes are likely to precede cell lysis and may go undetected, using these insensitive markers. One potentially significant and sensitive metabolic alteration resulting from oxidative vascular injury is the loss of prostacyclin (PGI₂) synthetic capacity. Impaired capacity to form this prostaglandin may contribute to atherogenesis and aging in that PGI₂ is known to be a potent vasodilator (10), an inhibitor of platelet aggregation and adherence (11, 12), a cytoprotective agent (13), a regulator of cholesterol ester hydrolase activity (14), and an inhibitor of smooth muscle cell proliferation (15).

Reduced PGI₂ synthetic capacity has in fact been observed to occur in atherosclerosis (16, 17) and with aging (18), and may be mediated at least in part by the overproduction of free radicals (8, 18), or by the loss of protection against reactive oxygen species. The metabolism of endogenous or exogenous arachidonic acid by cyclooxygenase in seminal vesicle preparations (19), cultured endothelial cells (20), or intact perfused blood vessels (21) generates radicals which cause the rapid inactivation of the enzymes required to convert arachidonic acid to PGI₂. In the perfused rabbit aorta, we have demonstrated that both cyclooxygenase and PGI₂ synthase are extremely sensitive to oxidative metabolites generated during arachidonic acid metabolism or during the reduction of exogenous 15-hydroperoxyarachidonate (21). The sensitivity of this enzyme system to oxidative stress, and the fact that the pattern of enzymatic inactivation is dependent on the type of oxidative

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1. *Abbreviations used in this paper:* GCMS, gas chromatograph-mass spectrometer; HBS, Hanks' balanced salts; HPLC, high performance liquid chromatography; PG, prostaglandin; PGE₂, prostaglandin E₂; PGF_{1 α} , 6-keto-prostaglandin F_{1 α} ; PGH₂, prostaglandin cyclic endoperoxide; PGI₂, prostacyclin; LDH, lactate dehydrogenase.

metabolite present (21), suggested to us that inhibition of arachidonic acid cascade enzymes might be a very sensitive indicator of oxidative endothelial cell injury and might approximate more closely the result of those oxidative stresses *in vivo* that result in subtle cellular injury but not in gross cell membrane disruption.

To address this hypothesis, we have examined the effects of oxidative stress on the metabolism of arachidonic acid in cultured endothelial cells and have compared these results with those obtained by employing ^{51}Cr release, LDH release, and cell morphology as end points of oxidative injury. We have used H_2O_2 as the injurious agent because endothelial cells have been shown to be particularly sensitive to H_2O_2 -induced injury.

Methods

Materials. Arachidonic acid, xanthine, xanthine oxidase, superoxide dismutase, catalase, glucose oxidase, and type I collagenase were purchased from Sigma Chemical Co., St. Louis, MO. Culture medium (medium 199 with Hanks' salts and phenol red indicator), Hanks' salts with and without Mg^{2+} and Ca^{2+} , antibiotic/antimycotic mixture (10,000 U of penicillin, 10,000 μg of streptomycin, and 25 μg of fungizone/ml), trypsin (0.5 g/liter)-EDTA (0.2 g/liter) solution, and sterile sodium bicarbonate (7.5%) were obtained from Grand Island Biological Co., Grand Island, NY. Fetal calf serum was obtained from HyClone Laboratories, Logan, UT. Ionophore A23187 was obtained from Calbiochem-Behring Corp., San Diego, CA. Hydrogen peroxide (30% solution, analytical grade) was obtained from Mallinckrodt, Inc., St. Louis, MO. Radiolabeled chromium [^{51}Cr]sodium chromate and [5,6,8,9,11,12,14,15- ^3H]arachidonic acid were obtained from New England Nuclear, Boston, MA; [3,3,4,4- ^2H]6-keto-prostaglandin $\text{F}_{1\alpha}$ ($\text{PGF}_{1\alpha}$) from the Upjohn Co., Kalamazoo, MI, and [3,3,4,4- ^2H]prostaglandin E_2 (PGE_2) from Merck Sharp & Dohme, Montreal, Quebec. All solvents were of high performance liquid chromatography (HPLC) grade and all derivatizing reagents were of the highest quality available.

Prostaglandin (PG) analysis. Deuterated internal standards (600–800 ng) were added to media collected from cell cultures after an experiment. Samples were acidified to pH 3 with formic acid and PGs extracted with ethyl acetate (1 vol three times). Combined extracts were evaporated to dryness under N_2 at 30–40°C, redissolved in 0.1 ml of methanol, and treated with ethereal diazomethane (0.2 ml) for 5 min to form the methyl ester. After evaporation to dryness, the esters were treated with a saturated solution of methoxyamine HCl in pyridine (0.3 ml overnight at room temperature) and evaporated to dryness, and the methyl ester-methoxime extracted from the residue with diethyl ether. Samples were further derivatized by reaction with 20 μl of pyridine/*N,O*-bis-[trimethylsilyl]-trifluoroacetamide (Pierce Chemical Co., Rockford, IL) (1:1) for 2 h at room temperature. The fully derivatized samples were evaporated to dryness and redissolved in hexane before analysis by selected ion monitoring (22) using a gas chromatograph-mass spectrometer ([GCMS] model 5992B; Hewlett-Packard, Palo Alto, CA) operated with the gas chromatograph oven at 250°C using a 6-ft \times 4-mm i.d. glass column packed with 3% OV-1 on 80–100 Supelcoport (Supelco, Inc., Bellefonte, PA) and a helium carrier gas flow of 25 ml/min. The ion source and jet separator were maintained at 260°C. Monitored ions (endogenous vs. deuterated) were mass/charge (*m/z*) 598 vs. 602 and 508 vs. 512 for the methyl ester-methoxime-trimethyl silyl ethers of 6-keto- $\text{PGF}_{1\alpha}$ and PGE_2 , respectively. Under the conditions above, the PGE_2 derivative (both major and minor methoxime isomers) was completely resolved from the 6-keto- $\text{PGF}_{1\alpha}$ derivative by the OV-1 column so that no contribution of ions from the 6-keto- $\text{PGF}_{1\alpha}$ derivative was found in the spectrum of the PGE_2 derivative. Thus the compounds were quantitated in the same injection.

Cell culture. Endothelial cells were collected from porcine aortas and cultured as described by Jaffe et al. (23). Briefly, freshly collected aortas were cleaned, filled with 0.1% collagenase in medium 199, sealed with clamps, and incubated at 37°C for 15–20 min. Freed cells were collected, washed by centrifugation at 4°C, and resuspended in medium 199 containing Hanks' salts, 10% fetal calf serum, and 1% antibiotic antimycotic mixture. Cells were then plated (400,000/flask) in 25-cm² flasks (Costar, Cambridge, MA) and monitored until ~50% of the cell clumps adhered. Nonadhering cells were poured off and the primary isolates were incubated in medium 199 plus 10% fetal calf serum at 37°C. The medium was changed every 3 d as the cells grew to confluence. Porcine aortic endothelial cells in culture were polygonal in shape and demonstrated contact inhibition. Further identification was as previously published (24). Factor VIII antigen was demonstrated in random cultures on 90–95% of the cells. The major contaminant, smooth muscle cells, did not reach a significant level during early passage (<5th passage). Primary and subsequent cultures were treated with 0.1% trypsin-EDTA at 37°C and split 1:4 for subculture. In general, confluent cultures in the first or second passage were used, although cells have been studied after the fifth passage with no apparent difference in response. Except where noted, all incubations were carried out in air at 37°C in Hanks' salts solution at pH 7.4. No change in cell morphology or pH of the medium was observed under these conditions.

H_2O_2 concentrations were determined in culture medium by colorimetric procedures essentially according to methods published by Thurman et al. (25). Aliquots of the medium were added to 0.1 ml of 25% trichloroacetic acid, 0.2 ml of 10 mM ferrous ammonium sulfate, and 0.1 ml of 2.5 M potassium thiocyanate. The absorbance at 480 nm was determined against a blank containing no sample. LDH was measured by determining first-order rate of NADH oxidation spectrometrically at 340 nm using pyruvate and NADH (26). Protein was measured using the Coomassie blue dye method (27).

To study the effects of H_2O_2 on endothelial cells in culture, monolayers were washed (to remove medium 199 and serum) and incubated in Hanks' balanced salts (HBS) containing varying doses of H_2O_2 . The buffer containing H_2O_2 was then poured off and used to determine the H_2O_2 concentration. The cells were washed again and incubated with arachidonic acid (5 μM) for 3 min at 37°C. The medium was then collected, internal standards were added, and the amount of PGs was determined. The effects of superoxide were determined after incubating cells in HBS containing either xanthine (5 mM), xanthine oxidase (1 U/ml), or both for 15 min at 37°C. Superoxide dismutase (5 U/ml) and catalase (5 U/ml) were added along with xanthine and xanthine oxidase in selected experiments. After incubation the medium was poured off and analyzed for H_2O_2 concentration. Fresh HBS containing arachidonate (5 μM) was added and PGI_2 production determined as above.

To study the effects of H_2O_2 on cell integrity, either ^{51}Cr or LDH release was determined in cells treated with peroxide. Cells were prelabeled by incubating confluent monolayers with 2 μCi of [^{51}Cr]sodium chromate for 3 h. The monolayers were washed to remove extracellular [^{51}Cr] and fresh HBS with or without H_2O_2 added. Aliquots were taken at various times and the amount of ^{51}Cr released was determined by using a gamma counter. After the experiment the cells were lysed in 0.2% sodium dodecyl sulfate and the amount of label remaining was determined. To measure the release of LDH, cells were incubated in HBS that contained H_2O_2 . After various times the buffers were removed, the monolayers were washed, and LDH released into fresh HBS during a subsequent 15-min incubation was determined. LDH remaining in the cells was determined after lysing the cells in distilled water and freezing and thawing.

To examine the effects of H_2O_2 on phospholipase activity, endothelial cells were prelabeled by incubation overnight with 2 μCi /flask of [^3H]arachidonate (24). The cells were washed to remove unincorporated label and incubated in HBS containing H_2O_2 . After pouring off the H_2O_2 , cells were stimulated with ionophore A23187 (1 μM in buffer containing 0.5% bovine serum albumin). The amount of label released

(primarily [^3H]arachidonate, reference 24) was quantitated by liquid scintillation spectrometry. The pattern of distribution of label into various labeled products was determined by HPLC (24). Briefly, labeled products were extracted from the incubation buffer as described above and applied to a reverse-phase HPLC column (μ -Bondapak Phenyl; Waters Associates, Millipore Corp., Milford, MA). PGs were eluted with 23% acetonitrile and 0.1% acetic acid in H_2O for 40 min. Arachidonic acid was then eluted by a linear gradient from 23 to 90% acetonitrile and 0.1% acetic acid in H_2O . Flow rates were 1.0 ml/min and 1-min fractions were collected and sampled to determine distribution of label into individual compounds.

The effects on cyclooxygenase and PGI_2 synthase activity were determined by incubating cells with either arachidonic acid, ionophore A23187, or prostaglandin cyclic endoperoxide (PGH_2) and measuring the quantity of PGI_2 and PGE_2 formed by GCMS procedures (see above). PGH_2 was prepared as previously described (18). The amount of total PG formed after incubation with arachidonate or ionophore A23187 represents combined cyclooxygenase and PGI_2 synthase activities whereas PGI_2 formed from PGH_2 represents PGI_2 synthase activity alone.

To test for significant differences, data were analyzed by one-way analysis of variance followed by the Student–Newman–Kuels test in cases where more than two means were compared.

Results

The effects of H_2O_2 on endothelial cell monolayers were studied by preincubating cultures with varying doses of peroxide. After this pretreatment, the ability of the cells to metabolize arachidonic acid to PGI_2 was determined. In these experiments H_2O_2 was found to inhibit potently the formation of PGI_2 by endothelial cells. The IC_{50} was approximately 35 μM (Fig. 1). This dose-response relationship within the range examined was not time dependent; i.e., preincubation of cells with H_2O_2 for 1 h did not produce any greater effect on PGI_2 production than preincubation for 1 min. The rapidity of the inhibition caused by H_2O_2 is further illustrated in Fig. 2. Again the maximum extent of inactivation was achieved within 1 min of pretreatment. To determine whether the time course observed might reflect rapid loss of H_2O_2 from the buffer above the monolayer, we measured the change in H_2O_2 concentration as a function of time. Compared with the time course for the inactivation of the prostaglandin cascade, the decrease in H_2O_2 concentration was much slower (Fig. 2). Approximately 30%

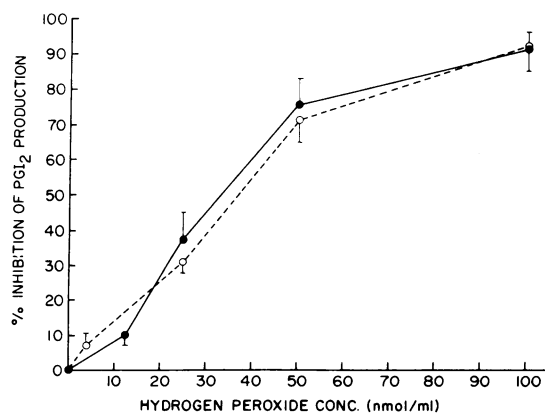


Figure 1. The effect of H_2O_2 on PGI_2 formation by porcine aortic endothelial cells. Cells were preincubated for 1 min (●) or 1 h (○) with H_2O_2 . After rinsing, the quantity of PGI_2 synthesized was determined in a subsequent 3-min incubation with 5 μM arachidonic acid. Data are presented as mean \pm SEM, $n = 6$.

of the original dose was present after 30 min of preincubation. Thus even after the maximum extent of inhibition of prostaglandin synthesis had occurred, significant levels of H_2O_2 remained.

The effects of H_2O_2 on cellular integrity were studied in endothelial cells prelabeled with ^{51}Cr or by measuring LDH release from cells pretreated with H_2O_2 . In dramatic contrast to the effects of H_2O_2 on PGI_2 synthesis, H_2O_2 produced both a time- and dose-dependent injury to treated cells leading to cell membrane disruption; H_2O_2 doses of at least 0.5 mM and incubation times in excess of 1 h were required before sufficient damage was inflicted to allow ^{51}Cr release (Fig. 3). In fact, doses of H_2O_2 up to 1 mM did not significantly increase release until after 60 min. At 0.1 mM H_2O_2 , a dose that completely inhibited PGI_2 formation (Fig. 1), no significant change in ^{51}Cr release could be seen even after a 2-h incubation (Fig. 3). Thus H_2O_2 -induced damage of endothelial cells exhibits a biphasic response. Low concentrations very rapidly inhibit a potentially important function of these cells (PGI_2 formation) whereas 10–20-fold higher concentrations and at least 60-fold longer exposure are necessary to produce damage sufficient to cause cell lysis.

Similar effects on LDH release were observed. Cells were preincubated with H_2O_2 and LDH release determined during a subsequent incubation in medium without H_2O_2 (Fig. 4). Again, incubation times as long as 60 min were required to see significant changes in LDH release. Additionally, as observed with ^{51}Cr release, a sharp decrease in cellular content (increased release) occurred at 1 h. However, in contrast to the ^{51}Cr data (Fig. 3), H_2O_2 at 1 mM appeared to reduce total cellular LDH rapidly (60% of total intracellular LDH remaining after 30 min). This loss may represent release during the preincubation with H_2O_2 or inactivation of intracellular LDH by radicals produced in response to this high dose of peroxide.

Endothelial cell injury was also studied in monolayers incubated with the superoxide generating system, xanthine plus xanthine oxidase. In these experiments (Fig. 5), preincubation with xanthine (0.5 mM) alone had no effect on subsequent conversion of arachidonic acid to PGI_2 . Xanthine oxidase (0.1 U/ml) produced a modest inhibition of PGI_2 synthesis (35%), perhaps due to the presence of endogenous endothelial xanthine or hypoxanthine (28) or to proteases that may contaminate xanthine oxidase preparations. Xanthine oxidase plus exogenous xanthine inhibited PGI_2 formation by 88% (Fig. 5). Cells were not protected from these effects by co-incubation of monolayers with the superoxide-generation system plus superoxide dismutase (5 U/ml). However, the inactivation PGI_2 synthesis was prevented by co-incubation of the generating system with catalase (5 U/ml) or by co-incubation of the generating system with catalase and superoxide dismutase. Thus it appears that these cells are damaged by H_2O_2 but not directly by superoxide. Neither boiled xanthine oxidase, catalase, nor superoxide dismutase alone had any effect on the conversion of arachidonate to PGI_2 (data not shown). When the concentration of H_2O_2 was assayed in the medium above the cell monolayers treated with xanthine plus xanthine oxidase, significant quantities were detected (Fig. 6). As is evident from the data given in Fig. 6, PGI_2 formation decreased with increasing production of H_2O_2 . In a similar system, H_2O_2 generated by glucose oxidase (0.1 U/ml) plus glucose (25 mM) was effective in inhibiting the conversion of arachidonate to

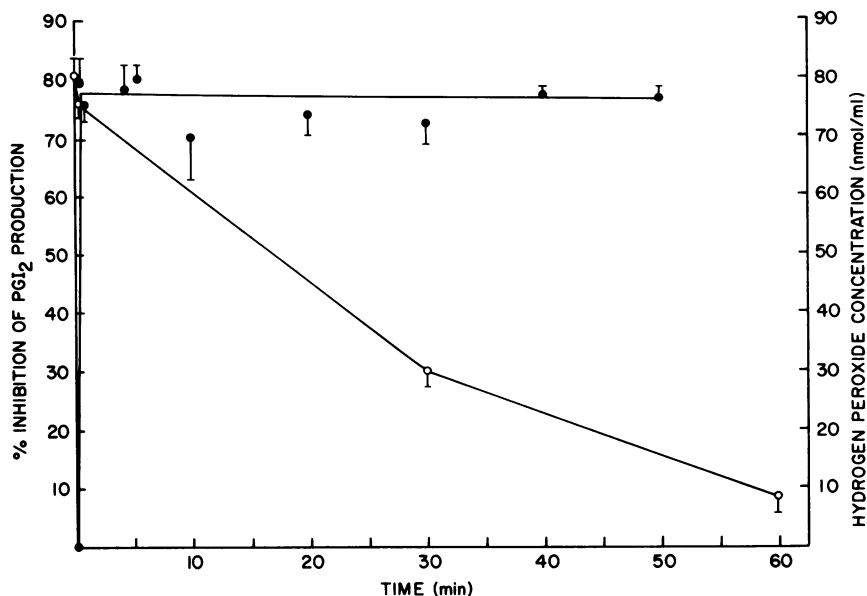


Figure 2. PGI₂ synthesis and H₂O₂ degradation by endothelial cell monolayers as a function of time. Cells were preincubated with H₂O₂ (50 μ M), rinsed, and subsequently incubated with arachidonic acid to determine the extent of inhibition of PGI₂ formation (●) (see Fig. 1). H₂O₂ concentrations (○) were determined in the medium above the monolayers at the times indicated. Data given as mean \pm SEM, $n = 6$ (●) and $n = 3$ (○).

PGI₂ (98%, data not shown) and again catalase (5 U/ml) was completely protective.

We next determined which enzyme(s) in the pathway from phospholipid to PGI₂ might be inhibited by H₂O₂. Although inactivation of phospholipase seemed unlikely in that a similar inhibition of PGI₂ synthesis was achieved when either exogenous arachidonic acid or ionophore A23187 was used to stimulate formation of PGI₂ (Fig. 1 and Table I; $\sim 50\%$ with 50 μ M H₂O₂), we investigated the effects of H₂O₂ on this enzyme and on cyclooxygenase and PGI₂ synthase. To study changes in phospholipase activity, endothelial cell phospholipids were prelabeled by incubation overnight with [³H]arachidonate (1 μ Ci/10⁶ cells). Under these conditions $\sim 70\%$ of the label was incorporated into cellular lipids, 92% of which was found in phospholipids (24). Preincubation of labeled cells with a dose of H₂O₂ (50 μ M) which inhibited PGI₂ synthesis by $\sim 50\%$ (Fig. 1), had no discernible effect on phospholipase activation by ionophore A23187 (Fig. 7); i.e., equivalent amounts of

labeled products were released from control and H₂O₂-treated cells both basally and after ionophore stimulation. 15 min after stimulation with ionophore A23187, labeled products released from control (non-H₂O₂-treated) cells were analyzed by HPLC and found to be composed primarily of arachidonic acid (63%, $n = 3$), PGI₂ (21%), and PGE₂ (16%). Labeled products released from H₂O₂-treated cells included arachidonic acid (82%, $n = 3$), PGI₂ (11%), and PGE₂ (7%). Although total amount of label released was the same for both treatments, the amounts of PGI₂ and PGE₂ formed decreased by $\sim 51\%$ each. As both PGI₂ and PGE₂ are derived from cyclooxygenase intermediates, a similar decline in both indicates inhibition of cyclooxygenase with no inhibition of PGI₂ synthase. This was confirmed by incubating control and H₂O₂-treated cells with arachidonic acid, ionophore A23187, or PGH₂ and measuring the amount of PGI₂ and PGE₂ produced. As shown in Table I, PGI₂ and PGE₂ formation from either arachidonic acid or ionophore A23187 was inhibited similarly by preincubation with H₂O₂ (59% and 65%, respectively). However, H₂O₂ pretreatment did not inhibit conversion of PGH₂ to PGI₂ by PGI₂

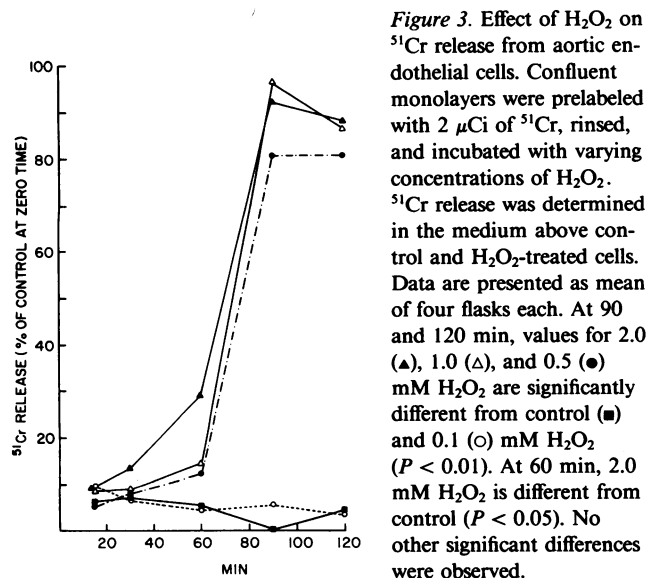


Figure 3. Effect of H₂O₂ on ⁵¹Cr release from aortic endothelial cells. Confluent monolayers were prelabeled with 2 μ Ci of ⁵¹Cr, rinsed, and incubated with varying concentrations of H₂O₂. ⁵¹Cr release was determined in the medium above control and H₂O₂-treated cells. Data are presented as mean of four flasks each. At 90 and 120 min, values for 2.0 (\blacktriangle), 1.0 (\triangle), and 0.5 (\bullet) mM H₂O₂ are significantly different from control (\blacksquare) and 0.1 (\circ) mM H₂O₂ ($P < 0.01$). At 60 min, 2.0 mM H₂O₂ is different from control ($P < 0.05$). No other significant differences were observed.

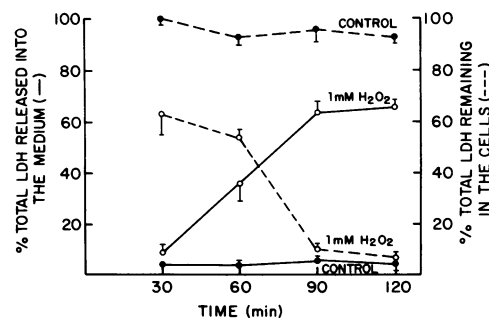


Figure 4. Effect of H₂O₂ on LDH release from aortic endothelial cells. Confluent monolayers were preincubated with H₂O₂ for the times indicated and rinsed to remove the peroxide. Fresh buffer was added and LDH activity remaining in the monolayers or released into the buffer determined after an additional 15-min incubation. Data are given as mean plus or minus range.

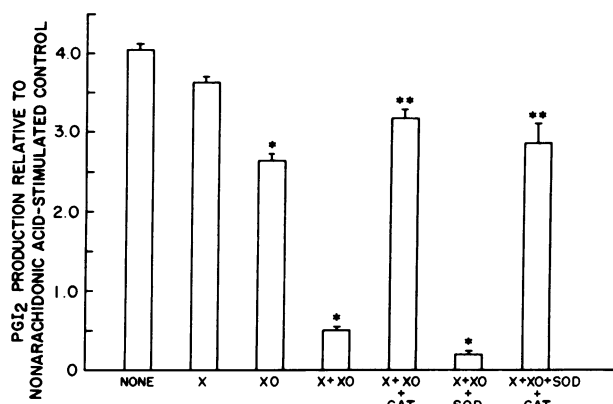


Figure 5. The effect of a superoxide generating system, xanthine (X) plus xanthine oxidase (XO), on PGI₂ formation by endothelial cells. Monolayers were preincubated for 30 min with buffer alone, xanthine (0.5 mM), xanthine oxidase (0.02 U/ml), catalase (CAT) (5 U/ml), or superoxide dismutase (SOD) (5 U/ml) in the combinations given in the figure. After rinsing, the cells were incubated for 3 min with 5 μ M arachidonic acid, and the amount of PGI₂ formed was determined in the medium above the cells. Data are given as mean \pm SEM, $n = 10$ (first four bars) and $n = 6$ (last three bars). * $P < 0.01$ as compared with no additions; ** $P < 0.01$ as compared with xanthine plus xanthine oxidase or xanthine plus xanthine oxidase and superoxide dismutase.

synthase. Thus, in endothelial cell monolayers, H₂O₂ appears to inhibit cyclooxygenase but not PGI₂ synthase.

Pretreatment of endothelial cells with H₂O₂ also prevented conversion of endogenous arachidonate to PGI₂. When monolayers were preincubated for 10 min with 50 μ M H₂O₂, ionophore A23187-stimulated PGI₂ production was inhibited by 59% whereas ionophore A23187-stimulated PGE₂ production was inhibited by 65%. Basal production of PGI₂ was inhibited by 28%, although this reduction did not reach significance. Basal production of PGE₂ was inhibited by 67% ($P < 0.05$) (Table I).

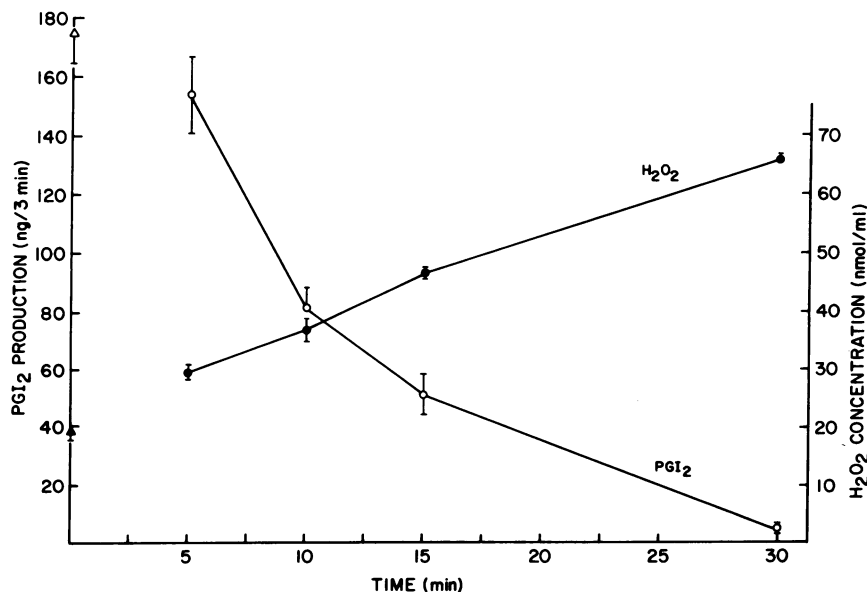


Figure 6. The relationship between formation of H₂O₂ by xanthine plus xanthine oxidase and the extent of inhibition of PGI₂ formation as a function of time. Cells were preincubated for the times given with xanthine plus xanthine oxidase, rinsed, and incubated with arachidonic acid as described in Fig. 5. Data on the ordinate represent basal PGI₂ production (Δ) and PGI₂ synthesis in response to 5 μ M arachidonic acid (\blacktriangle). Data are given as mean \pm SEM, $n = 3$.

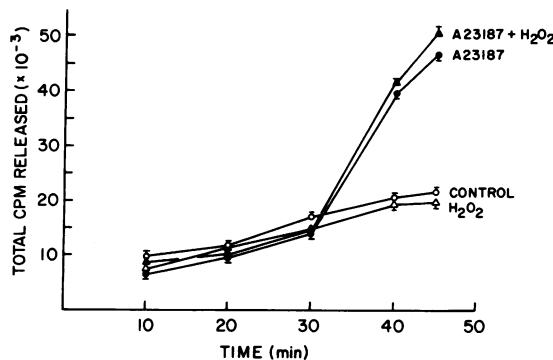


Figure 7. The effect of H₂O₂ on ionophore A23187 stimulation of arachidonic acid release. Endothelial cells were prelabeled by incubation with [³H]arachidonate. Labeled cells were then preincubated for 10 min with 50 μ M H₂O₂, rinsed, and fresh buffer added. Labeled products released were determined in aliquots of medium taken at 10, 20, 30, 40, and 50 min. Ionophore A23187 (1 μ M) was added to the flasks indicated at 30 min to stimulate phospholipase activity. Data are given as mean \pm SEM, $n = 6$.

Discussion

In these experiments we have shown that vascular endothelial cells are very sensitive to damage by H₂O₂. H₂O₂ potently blocked PGI₂ formation presumably by inhibiting cyclooxygenase. This effect occurred at a dose (35 μ M) and time course (>1 min) much different from that seen for cell membrane disruption. When either ⁵¹Cr or LDH release was monitored, doses greater than 500 μ M H₂O₂ and exposures of 60 min were necessary to produce the increases indicative of cell membrane damage. Similar inhibition of PGI₂ formation was also observed when cells were incubated with the superoxide-generating system, xanthine plus xanthine oxidase. The damaging species produced by this generating system appeared to be H₂O₂ and not superoxide itself in that catalase but not superoxide dismutase was protective.

Over the dose range studied, H₂O₂ produced a rapid inhibition of cyclooxygenase. Maximum inhibition was achieved

Table 1. Prostaglandin Formation

Buffer	PGI ₂ ng/10 min	PGE ₂ ng/10 min
Control	7.6±0.2	6.1±0.4
Ionophore A23187 (0.5 µM)	116±2	108±6
PGH ₂ (5 µg/ml)	61.9±0.8	2,180±30‡
H ₂ O ₂ (50 µM)	5.5±0.3	2.0±0.3
H ₂ O ₂ + ionophore A23187	51.7±1.4*	41.5±2.1*
H ₂ O ₂ + PGH ₂	75.2±1.3	2,190±56‡

Confluent monolayers of endothelial cells were pretreated with buffer or buffer containing 50 µM H₂O₂ for 10 min and then rinsed. Additions as indicated were made and the amount of PGI₂ and PGE₂ produced during a 3-min incubation period determined by GCMS analysis. Data are given as mean±SEM, *n* = 7.

* Values significantly different from ionophore A23187 alone (*P* < 0.01).

‡ Represents nonenzymatic breakdown of excess PGH₂.

within 1 min. No further effects were seen although H₂O₂ concentrations remained relatively high during the first 30 min of incubation (30% of initial concentration). In those experiments in which xanthine plus xanthine oxidase was used (see Fig. 6), progressive inhibition of PGI₂ formation accompanied accumulation of H₂O₂ in the medium. Based on the rapid time course of PGI₂ inhibition noted after exposure of endothelial cells to fixed concentrations of H₂O₂ (Fig. 2), the progressive decrease in cyclooxygenase activity seen in Fig. 6 would appear to be due to the progressively increasing levels of H₂O₂. The kinetics of H₂O₂-induced damage must be exceedingly complex in exhibiting both early events (cyclooxygenase inactivation) and late events (membrane disruption) with the exact time course being governed by actual intracellular H₂O₂ concentrations in the face of falling extracellular levels. In addition, if the ultimate toxic agent is the hydroxyl radical (OH•), then regulation of its formation by peroxidases and by the availability of Fe²⁺ would be important. The system is obviously even more complicated because susceptibility and kinetics may vary among individual compartments within the cell. Interestingly, endothelial cells have been shown to contain two distinct pools of cyclooxygenase which respond differently to exogenous versus endogenous arachidonate (29). Finally the role of protective systems such as catalase and the glutathione redox cycle (3, 30) in regulating peroxide levels must be considered.

In contrast to reports by others (31) that demonstrate either direct stimulation of PGI₂ formation by H₂O₂, or H₂O₂-mediated stimulation of PGI₂ formation by activated neutrophils (32), we found no stimulatory effects of H₂O₂ on basal production of PGI₂ by resting monolayers (Table I). Different experimental procedures could conceivably result in large variations in endogenous peroxide levels and therefore major differences in cellular peroxide tone. It is well known that such differences in peroxide tone can lead to significant differences in cellular metabolism of arachidonic acid (33–35), ultimately resulting in stimulation of cyclooxygenase activity as previously described (31, 32, 35) or in inhibition of this enzyme as our present data indicate. The ambient peroxide level in a cell is determined by a number of factors including the glutathione redox cycle (3, 30). The kinetics and capacity of this system to detoxify peroxides may ultimately determine the overall

concentration and thus effect of exposure to radical generating species. In previous studies in isolated perfused rabbit aorta, we have in fact observed both stimulation of prostaglandin production by low doses of 15-hydroperoxyarachidonate (2 µg/ml) and inhibition by higher concentrations (10 µg/ml) (18). Additionally, a regulatory role for lipid peroxides has been proposed where constant low levels of these compounds are required for cyclooxygenase activity (35). Therefore it may be difficult to compare data between experiments where exogenous peroxides are added if the status of endogenous protective systems is unknown.

H₂O₂ may also have other effects on intact cells which lead to elevated prostaglandin synthesis. For example, in a recent report (31), H₂O₂ treatment results in rapid disturbance in K⁺ flux with a time course which resembles the pulse of prostaglandin synthesis described. The effect of H₂O₂ on the flux of other ions such as Ca²⁺ has not been investigated. A net Ca²⁺ influx occurring along with K⁺ efflux would certainly activate phospholipase activity, arachidonic acid release, and subsequent prostaglandin synthesis.

Metabolism of endogenous or exogenous arachidonic acid leads to inactivation of both cyclooxygenase and prostacyclin synthase, presumably due to overproduction of free radicals (19, 36). Of the two enzymes, cyclooxygenase, on the one hand, appears to be particularly sensitive to such inhibition. On the other hand, addition of exogenous lipid peroxides (15-hydroperoxyarachidonate) preferentially inactivates PGI₂ synthase (18). The exact mechanism for inactivation of these enzymes is unknown. However, activity of the peroxidase component of the cyclooxygenase appears to be involved (19). Enzymatic reduction of prostaglandin G₂ to PGH₂ by this peroxidase has been shown to produce a radical which is destructive towards cyclooxygenase activity (19). Although the identity of this radical remains controversial (37), the highly reactive OH• has been implicated (19, 38). Production of OH• radicals may also be involved in the inactivation of cyclooxygenase by H₂O₂. Certainly OH• can be easily produced from H₂O₂ by several routes including the Fenton reaction catalyzed by Fe²⁺ (5), by heme proteins, and possibly by the peroxidase component on cyclooxygenase itself (37, 39). In addition, secondary radicals such as lipid radicals may also play a role in these effects. Inhibition by H₂O₂ appears to involve covalent destruction of enzyme protein as recovery of activity requires 2–4 h of incubation in complete medium and is inhibited by protein synthesis inhibitors (Barchowsky, A., A. R. Whorton, and R. S. Kent, unpublished data).

In the present study, exogenous H₂O₂ inhibited cyclooxygenase exclusively with no measurable effect on PGI₂ synthase. It is possible that the polarity of the parent peroxide dictates which enzyme is inactivated by radical attack. This may be determined in part by unique requirements for the interaction of various peroxides with the enzyme protein. Alternatively, compartmentation and availability of these enzymes to peroxides of differing polarity may be important. From the present study and from our previous work, it appears that polar peroxides such as H₂O₂ selectively inhibit cyclooxygenase whereas lipophilic peroxides such as 15-hydroperoxyarachidonic acid selectively inhibit PGI₂ synthase particularly at low levels. Thus differential inactivation of the enzymes of the arachidonic acid cascade by various peroxides may have important regulatory effects on both the amount and pattern of prostaglandins produced by a tissue.

In a recent report (31), H_2O_2 was shown to block specifically ionophore A23187-stimulated PGI_2 production. This effect was found only to occur at high O_2 tension. No effect of 100 μM H_2O_2 was seen on cells incubated in air, and no information regarding the site of inhibition was given. From these data it is not possible to determine potential inhibitory effects of H_2O_2 on phospholipase activity. In contrast, our results from experiments done with cells incubated in air indicate that H_2O_2 inhibits ionophore A23187-stimulated PGI_2 production at similar doses and under similar conditions as found for inhibition of the cyclooxygenase. However, because we found no alteration in the amount of labeled products released after ionophore A23187 stimulation, an effect of H_2O_2 phospholipase activity is doubtful.

As has been shown in a number of studies, endothelial cells are directly damaged by H_2O_2 (1-3). These cytolytic actions of H_2O_2 require treatment for up to 60 min at doses in the range of 1 mM. Data from our experiments on the release of ^{51}Cr suggest that a critical level of damage must be reached and that beyond this threshold ^{51}Cr release increases rapidly. Thus endothelial cells appear to be able to protect their membranes from damage resulting from high levels of peroxides for up to 60 min. Other cytotoxic effects that are more subtle and not lethal are evident at lower levels of H_2O_2 and after shorter exposures. For example, ~40% of intracellular LDH activity is lost during the first 30 min of treatment with 1 mM H_2O_2 . Doses of H_2O_2 below 100 μM have also been observed to stimulate purine release and K^+ efflux from endothelial cells with a time course of a few minutes (31). In addition, the capacity to synthesize PGI_2 is rapidly lost at low doses of H_2O_2 ($IC_{50} = 35 \mu M$). This may be particularly significant in a variety of vascular conditions including atherosclerosis and aging where oxidative cellular damage may be severe enough to inhibit arachidonic metabolism, yet not result in cell membrane disruption and death.

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