DIFFERENTIAL EFFECTS OF HYDROGEN PEROXIDE ON INDICES OF ENDOTHELIAL CELL FUNCTION

By ANN AGER* and JOHN L. GORDON[‡]

*From the *Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT and the [‡]Section of Vascular Biology, Medical Research Council Clinical Research Centre, Harrow, Middlesex HA1 3UJ, United Kingdom*

Interactions of granulocytes with vascular endothelial cells, a prominent feature of the inflammatory response, can result in endothelial damage. Hydrogen peroxide, released during the respiratory burst associated with granulocyte activation, has been implicated in causing lethal damage on the basis of experiments that demonstrated catalase-inhibitable release of ^{51}Cr from endothelial cells (1, 2). Activated granulocytes can also have sublethal effects on endothelium (for example, inducing transient increases in microvascular permeability [3]), but the functional responses of endothelium in such circumstances have not been investigated or quantified. To do this, it would be desirable to define sensitive indices of endothelial functions and to monitor such functions simultaneously with conventional measures of cell damage.

We therefore measured K^+ efflux (using ^{86}Rb), prostaglandin production, and the release of cytoplasmic purines from endothelial cells, as well as measuring cytotoxic effects by monitoring adenine uptake, ^{51}Cr release, and the uptake of vital dye. The damaging stimuli we used were reagent H_2O_2 and xanthine plus xanthine oxidase, which has been previously used as a cell-free model of the superoxide-generating system in the granulocyte (4). It is perhaps worth noting that the neutrophil respiratory burst can produce active agents other than those generated by xanthine oxidase (for example, products of myeloperoxidase and any compounds resulting from interactions between activated oxygen species and other neutrophil secretory products), although the major role in neutrophil-mediated endothelial destruction has been ascribed to H_2O_2 (2).

We confirmed that H_2O_2 was the product generated by xanthine plus xanthine oxidase that was mainly responsible for the effects on endothelium and we found that the first three of the parameters of cell function listed above were all affected by concentrations of H_2O_2 at least 30-fold lower than those causing lethal damage to the cells. In addition, exposure to even lower concentrations of H_2O_2 (<0.1 μM) prevented endothelial cells from responding to vasoactive agents.

Materials and Methods

Materials

Xanthine, hydrogen peroxide (30% solution), cytochrome c (type VI from horse heart), superoxide dismutase (type I, 2,700 U/mg protein), catalase (2,100 U/mg protein), thrombin (human, 3,000 NIH U/mg), bradykinin triacetate, adenosine triphosphate,

pyruvic acid, and α-nicotinamide adenine dinucleotide (NADH)[1] were all purchased from Sigma (London) Chemical Co. Trypsin (1:250) was obtained from Difco Laboratories, Detroit, MI, and calcium ionophore A23187 from Calbiochem-Behring, Bishops Stortford, UK. Xanthine oxidase (0.5 U/mg protein), prepared according to the method of Waud et al. (5), was a generous gift from Dr. H. W. Dougherty, Merck Institute for Therapeutic Research, Rahway, NJ. One enzyme unit equals 1 μmol substrate converted per min at pH 7.0 and 25°C. Krebs' solution of the following composition (mM) was used: NaCl, 119; KCl, 3.1; MgSO₄, 0.6; NaHCO₃, 25; KH₂PO₄, 1; CaCl₂, 1.3 and glucose, 11.1, gassed with 5% CO_2 in either oxygen or air to maintain the pH at 7.4.

Cell Culture

Aortic endothelial cells were cultured from 1–14-d-old pigs as previously described (6) and were used after 3–15 passages. Experiments to measure cytotoxicity were performed in quadruplicate on confluent monolayers of cells in 7-mm diam wells (~10⁴ cells/well) of tissue culture plates. After rinsing with phosphate-buffered saline (PBS) to remove growth medium and serum, cells were incubated with Krebs' solution (50 μl/well) with or without xanthine (100 μM), plus superoxide dismutase or catalase when indicated, for 5 min at 37°C in a humidified atmosphere of 5% CO_2 in air. Final concentrations of 0.001–0.3 U/ml xanthine oxidase were added at zero time and cells were incubated for a further 60 min. Experiments with reagent H_2O_2 (0.003–3.0 mM) were performed using the same protocol. The supernatant and/or cells were analyzed as described below.

Assessment of Cytotoxic Effects

Vital dye exclusion. After removing the test solutions, cells were incubated for 5 min with 0.1% trypan blue in PBS and vital dye uptake was monitored by phase-contrast microscopy. The percentage of cells in each field of view excluding trypan blue was determined by direct visual assessment.

⁵¹Chromium release. Endothelial cells were prelabeled for 18 h (overnight) with 50 μl/ well of 10 μCi/ml Na⁵¹CrO₄ (350–600 mCi/mg Cr; Amersham International, UK) in growth medium plus 20% (vol/vol) fetal calf serum (~200,000 cpm/well). Supernatants were removed and then the cells were washed three times with PBS to remove excess radioactivity and incubated with test reagents as described above. After 60 min the supernatants were collected and the cells solubilized with 0.2% Triton X-100. Total cell-associated ⁵¹Cr and ⁵¹Cr released into the supernatant were measured by gamma counting. Specific release was calculated from [(A-B)/(C + B)] × 100, where A is test release, B is spontaneous release, and C is cell-associated label. After 18 h incubation, 9.2 ± 0.2% (n = 14) of label was cell-associated and, during a subsequent 60 min incubation, 15.2 ± 1.5% (n = 14) of this incorporated label was released spontaneously. Results are expressed as the mean percentage (±SE) of control release.

[³H]adenine uptake. Adenine is actively transported into endothelial cells, and inhibition of adenine uptake after exposure to cytotoxic stimuli correlates with microscopic assessment of cell damage (7). After 60 min incubation with test reagents, the supernatants were removed and cells rinsed with PBS. 50 μl of 0.4 μM [2-³H]adenine (10 μCi/ml; 400,000 cpm) was added to each well and incubated for 30 min at 37°C. Cell-associated label was measured after extraction with 50 μl of 98% formic acid as previously described (7). After 30 min incubation with [³H]adenine, 45.7 ± 2.3% (n = 12) of label was cell-associated in control wells. Results are expressed as mean percentage of control uptake ± SE.

Assessment of Sublethal Effects

⁸⁶Rb efflux. Endothelial cells were plated at confluent density onto coverslips, 5 × 15 mm (~10⁵ cells), and used the following day. Cells were incubated for at least 2 h with 10 μCi/ml ⁸⁶Rb chloride (1–12 mCi/mg Rb; Amersham International) in growth medium

[1] *Abbreviations used in this paper:* LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; PG, prostaglandin; SOD, superoxide dismutase; TCA, trichloroacetic acid.

plus 20% fetal calf serum. Two coverslips were then placed back to back in a 3.5 ml perfusion chamber and the efflux of the isotope was monitored by perfusing with Krebs' buffer at 37°C, gassed with 95% O_2/5% CO_2 at a rate of 3.5 ml/min. Fractions were collected every 2 min, radioactivity in the fractions and cells was measured by gamma counting, and a first-order desaturation curve was plotted from the results as previously described (8). First-order rate constants (R) were plotted against time and expressed in units of min^{-1} ± SE.

After a steady rate of ^{86}Rb efflux had been obtained (>20 min), test solutions were applied for 4 min. Xanthine (100 μM) was mixed with xanthine oxidase (0.001–0.1 U/ml) immediately before testing. The change in ^{86}Rb efflux rate, ΔR, was calculated as the difference between the maximum efflux measured and the mean of five points immediately before the test solution was applied.

[^3H]purine release. Endothelial cells (1–2 × 10^6 cells) were seeded onto microcarrier beads (5 × 10^5 beads) (Biosilon; A/S Nunc, Roskilde, Denmark) in 70 ml of growth medium and stirred intermittently for 30 s every 30 min as previously described (9), until >50% of bead surface was covered. To visualize endothelial cells, an aliquot of beads was fixed in ethanol and stained with 0.1% methyl violet in H_2O. 0.2–0.5 ml of microcarrier beads were then packed into a 1-ml plastic syringe plugged with glass wool. The syringe was closed with a rubber-tipped plunger through which plastic tubing had been threaded (i.d. 0.5 mm) as previously described (10). Columns were perfused from below with Krebs' solution at 0.35 ml/min in a 37°C chamber and fractions were collected at 1-min intervals. To prelabel cells with [^3H]adenosine, columns were washed for 5–10 min and then 5 μCi/ml [2-^3H]adenosine (20–25 Ci/mmol; Amersham International) was recirculated for 60 min from a 5-ml reservoir. Previous studies (6) have shown that after 60 min this label is converted intracellularly to adenine nucleotides (mainly ATP). Excess radioactivity was removed by perfusing the columns for 15 min with Krebs' solution. Test solutions were infused for 4 min followed by a 16-min wash. Fractions were collected onto trichloroacetic acid (TCA) (final concentration, 5%) and aliquots (50 μl) were counted to determine total [^3H]purine release. Results are expressed as the ratio of stimulated ^3H release to basal ^3H release where basal release was defined as the mean of five fractions before initial exposure to test reagent. At the end of the experiment, cell-associated radioactivity was measured after solubilizing cells in 0.5 ml 0.2% Triton X-100. The profile of [^3H]purines was measured as previously described by thin-layer chromatography after neutralizing with 5 M K_2CO_3 (6, 11). Labeled columns usually contained 3–10 × 10^6 cpm and released ~0.15% of this total cpm/min^{-1} under basal conditions.

To assess cytotoxicity in these experiments, the release of lactate dehydrogenase (LDH) was measured as described by Wroblewski and La Due (12). Column fractions were made up to 1.0 ml in 0.2% Triton X-100 and cells were solubilized in 0.2% Triton. Results are expressed as a percentage of total LDH activity in cells.

Prostaglandin Production

Endothelial cells on microcarrier beads were packed into columns as described above and perfused for 60 min with Krebs' solution. Basal release of prostaglandin I_2 (PGI$_2$) and PGE$_2$ was then monitored by collecting 1-min fractions. Test solutions were applied for 4 min followed by a 16-min wash before the next test solution was applied, with a maximum of four tests per column. An aliquot of each fraction (20–50 μl) was taken for 6-keto-PGF$_{1\alpha}$ and PGE$_2$ radioimmunoassays as previously described (13). At the end of an experiment, the number of cells in each column were estimated by measuring DNA and/or protein. Results for [^3H]purine release and PG production are expressed as the increase over the mean basal level (calculated from the five points before applying the test solution).

Spectrophotometric Measurement of Oxygen Radical Production by the Xanthine/Xanthine Oxidase Reaction

Oxygen radical production was measured continuously by monitoring the increase in absorbance of cytochrome c at 550 nm using a double-beam spectrophotometer. The test cuvette contained 50 μM cytochrome c and 100 μM xanthine in 1 ml gassed Krebs'

solution at 37°C. Xanthine oxidase (0.001–0.3 U/ml) was added at zero time and absorbance was recorded. The reference cuvette was as the test cuvette, but with no enzyme added. E_{red-ox} of 21.1 mM^{-1} cm^{-1} was used to calculate the amount of cytochrome c reduced, and hence oxygen radicals produced, as previously described (14).

Results

Spectrophotometric Measurement of Oxygen Radical Production by the Xanthine/Xanthine Oxidase Reaction

The initial rate of cytochrome c reduction was dose dependent from 0.003 to 0.3 U/ml xanthine oxidase. The absolute amount of cytochrome c reduced was limited by its reoxidation; thus, a maximum response was obtained at 0.03–0.3 U/ml. At 0.03 U/ml xanthine oxidase, 20 μg/ml superoxide dismutase (SOD) (which catalyzes the dismutation of oxygen radicals to H_2O_2) inhibited cytochrome c reduction by 76.3%. The reoxidation of cytochrome c was abolished by catalase (which detoxifies H_2O_2); as a consequence, the total cytochrome c reduced by 0.03 U/ml was increased by 25.6% in the presence of 20 μg/ml catalase, from 19.9 to 25.1 nmol. The time taken for cytochrome c reduction and reoxidation (i.e., for absorbance to return to baseline) decreased progressively with increasing enzyme concentration, from 9 min at 0.01 U/ml to 2.1 min at 0.3 U/ml. Using E_{red-ox} of 21.1 mM^{-1} cm^{-1} for cytochrome, the maximum concentration of cytochrome c reduced was 21 μM at 0.03, 0.1, and 0.3 U/ml of enzyme.

Cytotoxic Effects of Hydrogen Peroxide and of Xanthine plus Xanthine Oxidase on Endothelial Cells

To establish the concentration dependence and time course of cytotoxicity induced by reagent H_2O_2 and by xanthine plus xanthine oxidase, we monitored ^{51}Cr release, vital dye uptake, and adenine uptake in cultures of pig aortic endothelial cells. Cells were incubated for 60 min with increasing concentrations of xanthine oxidase (0.01–0.3 U/ml) plus 100 μM xanthine, or of reagent H_2O_2 (10–1,000 μM). Xanthine oxidase (0.01–0.3 U/ml) induced no detectable cell damage whereas 60 min incubation with 300–1,000 μM H_2O_2 was cytotoxic. There were, however, marked differences in the extent of cytotoxicity measured by the three methods. In all experiments, 300–1,000 μM H_2O_2 caused >95% of the cells to take up trypan blue, but 300 μM and 1,000 μM H_2O_2 stimulated ^{51}chromium release only to ~120% of the control value; inhibition of adenine uptake after 60 min incubation with 300 μM and 1,000 μM H_2O_2 was 39% and 71%, respectively (Table I). Catalase (20 μg/ml) reduced release of ^{51}Cr induced by 300 μM H_2O_2 from 126 ± 10% to 93 ± 5% of the control value and returned adenine uptake to control levels.

For subsequent studies on sublethal effects of xanthine plus xanthine oxidase, or of reagent H_2O_2, enzyme concentrations of 0.01–0.3 U/ml and H_2O_2 concentrations of 3–100 μM were used routinely.

Sublethal Effects of Reagent H_2O_2 and of Xanthine plus Xanthine Oxidase on Endothelial Cells

Effect of xanthine plus xanthine oxidase on ^{86}Rb efflux. The first-order rate constant for ^{86}Rb efflux from pig aortic endothelial cells was 21.4 ± 1.2 × 10^{-3}

TABLE I

Cytotoxic Effects of Xanthine Plus Xanthine Oxidase, and of Reagent
Hydrogen Peroxide on Pig Aortic Endothelial Cells

Concentra-tion	^{51}Chromium release		Uptake of [^{3}H]adenine	
	XO	H_2O_2	XO	H_2O_2
1×	108 ± 12	95 ± 5	94 ± 6	103 ± 3
3×	113 ± 8	103 ± 3	105 ± 17	106 ± 1
10×	92 ± 10	94 ± 8	108 ± 5	98 ± 8
30×	104 ± 5	126 ± 10	112 ± 7	61 ± 9
100×	—	122 ± 6	—	29 ± 1

Cytotoxic effects were measured by release of ^{51}chromium and inhibition of [^{3}H]adenine uptake. Cells in static culture were treated as described in Materials and Methods with either xanthine oxidase (XO; 1× = 0.01 U/ ml) in the presence of 100 μM xanthine, or with H_2O_2 (1× = 10 μM). Results are expressed as the mean percentage of controls ± SE (n = 4); control release of ^{51}Cr was 2.8 ± 0.1 × 10^3 cpm (n = 8) and control uptake of [^{3}H]adenine was 1.8 ± 0.05 × 10^5 cpm (n = 8).
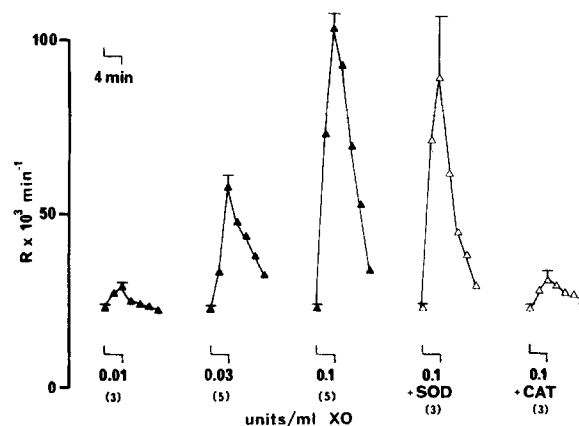

FIGURE 1. Stimulation of ^{86}Rb efflux from pig aortic endothelial cells by xanthine plus xanthine oxidase. Each point is the mean first-order rate constant (R), in units of min^{-1}. The first point of each peak represents mean basal efflux. Error bars represent SE and numbers of observations are shown in brackets below each peak. (▲) Experiments with xanthine oxidase; (△) experiments with xanthine oxidase plus either 20 μg/ml superoxide dismutase (SOD) or 20 μg/ml catalase (CAT). Xanthine (100 μM) was present in all experiments.

min^{-1} (n = 6). Xanthine plus xanthine oxidase infused for 4 min stimulated ^{86}Rb efflux in a dose-dependent manner: ΔR = 3.8 ± 0.5 × 10^{-3} min^{-1} at 0.01 U/ml; 33.7 ± 4.1 × 10^{-3} min^{-1} at 0.03 U/ml; and 79.5 ± 3.1 × 10^{-3} min^{-1} at 0.1 U/ ml (Fig. 1). In the absence of xanthine, the effect of 0.1 U/ml xanthine oxidase was reduced by ~95%. Efflux of ^{86}Rb returned to the control level upon removal of the stimulus. To determine whether oxygen radicals, hydrogen peroxide, or both, were responsible for stimulating ^{86}Rb efflux, the effects of SOD and of catalase were investigated. Each enzyme was added to the solution of xanthine (100 μM) before the addition of xanthine oxidase. SOD (20 μg/ml) had no significant effect on ^{86}Rb efflux induced by 0.1 U/ml xanthine oxidase, whereas catalase (20 μg/ml) inhibited efflux by 92% (Fig. 1), indicating that hydrogen

peroxide (and not oxygen radicals) was responsible for the stimulation of ^{86}Rb efflux induced by xanthine plus xanthine oxidase.

Xanthine plus xanthine oxidase also stimulated the release of [^{3}H]purines and PG production, which were both inhibited by catalase but not by SOD (results not shown). To explore the effects of H_2O_2 on endothelial cells in more detail, we used reagent H_2O_2 in subsequent studies of ^{86}Rb efflux, PG production, and [^{3}H]purine release.

Effects of reagent H_2O_2 on ^{86}Rb efflux and [^{3}H]purine release. Reagent H_2O_2 infused for 4 min stimulated ^{86}Rb efflux in a dose-dependent manner: $\Delta R = 6.9 \pm 2.1 \times 10^{-3}$ min^{-1} at 10 μM; 24.6 $\pm$ 3.0 $\times 10^{-3}$ min^{-1} at 30 μM; and 36.3 $\pm$ 4.6 $\times 10^{-3}$ min^{-1} at 100 μM ($n = 3$). Infusion of 100 μM H_2O_2 for 12 instead of 4 min did not increase the size of the response but efflux was maintained at a stimulated level throughout the infusion and returned to baseline upon removal of the stimulus (Fig. 2). Catalase (20 μg/ml) reduced the effect of 100 μM H_2O_2 by ~90%. For comparison, the effect of calcium ionophore A23187 (10 μM) is also shown (Fig. 2); this agent, previously shown to be particularly effective at stimulating ^{86}Rb efflux from pig aortic endothelial cells (10), produced a maximum ^{86}Rb efflux of 108.7 $\pm$ 6.4 $\times 10^{-3}$ min^{-1}. Unlike the response to prolonged infusion of H_2O_2, efflux started to return towards baseline levels in the continued presence of the stimulus.

Basal release of labeled purines from cells prelabeled with [^{3}H]adenosine was 0.15 $\pm$ 0.02% min^{-1} ($n = 7$, range 0.05–0.29%) of the total labeled pool. Reagent H_2O_2 infused for 4 min stimulated release in a dose-dependent way with a threshold of 10 μM (Fig. 3). Maximal stimulation was 2.2-fold at 10 μM H_2O_2, 34.9-fold at 30 μM H_2O_2, and 107.8-fold at 100 μM H_2O_2. The calcium ionophore A23187 also stimulated [^{3}H]purine release with a maximum of 62.0 $\pm$ 4.6-fold at 10 μM ($n = 3$).

Separation of labeled purines into nucleotides (ATP, ADP, AMP) and nucleosides (inosine, hypoxanthine, adenosine) by thin-layer chromatography showed that H_2O_2 stimulated the release of nucleosides. Basal release of [^{3}H]purines was 35 $\pm$ 2% nucleotides and 65 $\pm$ 3% nucleosides ($n = 8$). Exposure to H_2O_2 (10
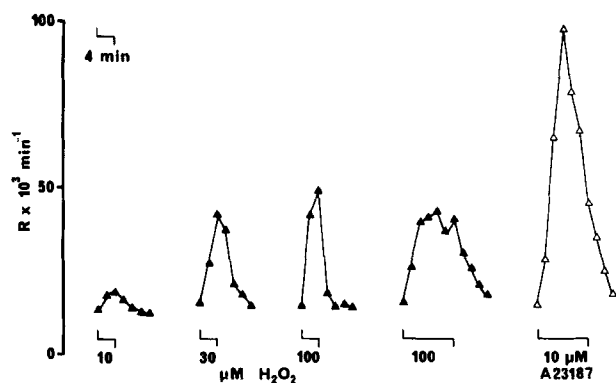

FIGURE 2.   Stimulation of ^{86}Rb efflux from pig aortic endothelial cells by hydrogen peroxide. Points show the first-order rate constant (R), in units of min^{-1}, for single observations made on the same cell strain. (▲) Experiments with H_2O_2 infused for 4–12 min; the response to 10 μM ionophore A23187 (infused for 12 min) is also shown (△).
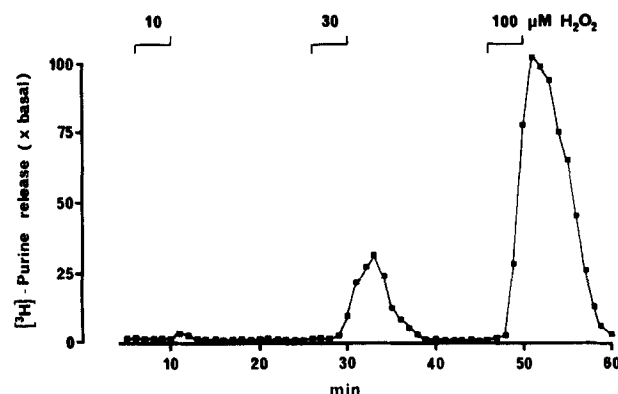
FIGURE 3. Stimulation of [^{3}H]purine release from pig aortic endothelial cells by hydrogen peroxide. Cells were prelabeled with [^{3}H]adenosine for 60 min as described in Materials and Methods, and H_2O_2 (10–100 μM) was infused for 4 min. Results are expressed as the ratio of stimulated ^{3}H release to basal ^{3}H release in a single representative experiment. Basal release was defined as the mean of five fractions before initial exposure to test reagent.
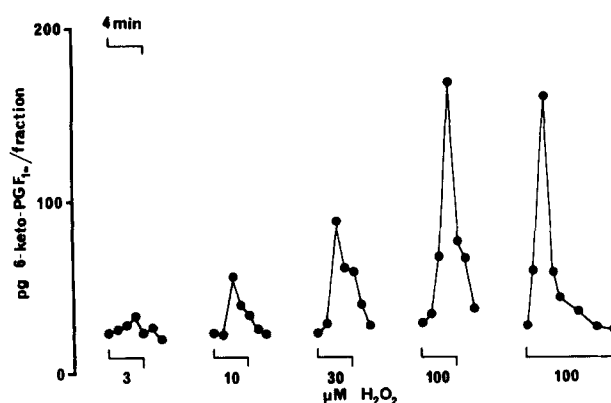
FIGURE 4. Stimulation by hydrogen peroxide of PGI_2 production by pig aortic endothelial cells. Points show 6-keto-$PGF_{1\alpha}$ (pg) produced per minute from superfused columns (see Materials and Methods) containing cells of the same strain. H_2O_2 (3–30 μM) was infused for 4 min; 100 μM H_2O_2 was infused either for 4–10 min.

and 100 μM) increased the proportion of nucleosides to 95%, whereas A23187 preferentially stimulated release of nucleotides (~80% of total ^{3}H released).

The release of LDH during infusion with either H_2O_2 or A23187 was not significantly different from normal. Basal release was 0.64 ± 0.05% ($n = 7$) and release of LDH at the peak of [^{3}H]purine release stimulated by 100 μM H_2O_2 was 0.65 ± 0.03% ($n = 4$).

Effects of reagent H_2O_2 on prostaglandin production. Basal release of PGI_2 measured by radioimmunoassay of its stable product, 6-keto-$PGF_{1\alpha}$, was 75 ± 3 pg column/min^{-1} ($n = 7$) (equivalent to 30–50 pg/10^6 cells/min). Fig. 4 shows the effect of increasing doses of H_2O_2, infused for 4 min, on PGI_2 production in one experiment. Reagent H_2O_2 stimulated PGI_2 production 2.2 ± 0.1-fold at 10 μM, 3.9 ± 0.3-fold at 30 μM, and 12.7 ± 1.8-fold at 100 μM H_2O_2 ($n = 10$). Infusion

of 100 μM H_2O_2 for 10 instead of 4 min did not affect the duration of the response; PGI_2 production had returned to the basal value within 6 min (Fig. 4). In addition to PGI_2, the production of PGE_2 by endothelial cells was similarly stimulated by H_2O_2 in a dose-dependent manner (results not shown).

Effects of reagent H_2O_2 on endothelial responses to vasoactive stimuli. Having established that H_2O_2 directly stimulated PGI_2 and PGE_2 production, we wished to determine whether prior exposure to H_2O_2 altered the responses of endothelial cells to other agents that stimulate PG production. Accordingly, responses to ATP, bradykinin, thrombin, trypsin, and ionophore A23187 were studied before and after exposure to H_2O_2.

Bradykinin (1 μM) stimulated PGI_2 production 2.7 $\pm$ 0.3-fold; thrombin (10 U/ml), 3.4 $\pm$ 0.2-fold; ATP (100 μM) 6.1 $\pm$ 0.5-fold; and A23187 (10 μM), 50 $\pm$ 9-fold (mean values $\pm$ SE; $n = 3$). All these agents stimulated PGE_2 production by a similar extent (results not shown).

When tested 20 min after exposure to 100 μM H_2O_2 (infused for 4 min), PGI_2 responses to all of the above agents were inhibited by 85–93%, as was the response to a second exposure of 100 μM H_2O_2. Further experiments revealed that PGI_2 production stimulated by these agents was affected by prior exposure to concentrations of H_2O_2 as low as 0.1 μM (Fig. 5). Production of PGE_2 in response to ATP and A23187 was measured after exposure to 100 μM H_2O_2; PGE_2 production was inhibited in parallel with PGI_2 responses.

Because enzymes involved in PG synthesis are known to be susceptible to inhibition by oxygen products, we investigated the effect of O_2 tension on the capacity of H_2O_2 to inhibit endothelial cell responses to stimuli. When endothelial cells on microcarrier beads were perfused with Krebs' solution bubbled in 95%
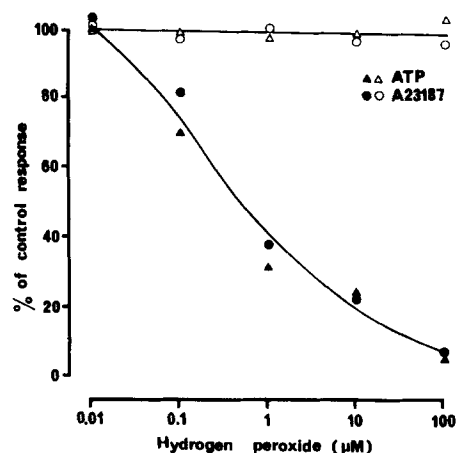

FIGURE 5. Inhibition of PG production stimulated by ATP and ionophore A23187 after exposure to hydrogen peroxide. Cells were exposed to increasing concentrations of hydrogen peroxide (0.01–100 μM) for 4 min and the response to either ATP (100 μM) or A23187 (10 μM) was measured 20 min later. Control responses to ATP and A23187 were measured on separate columns and results are expressed as percentages of this control response. Closed symbols ($\blacktriangle$, $\bullet$) are from experiments performed in Krebs' solution gassed with 95% O_2/CO_2; open symbols ($\triangle$, $\bigcirc$) are from experiments performed in Krebs' solution bubbled with 95% air/5% CO_2.

air/5% CO_2, rather than with 95% O_2/5% CO_2, PGI_2 responses to ATP and A23187 were no longer inhibited by prior exposure to H_2O_2 (Fig. 5). However, the capacity of H_2O_2 to stimulate PG production directly was unaffected (results not shown).

Discussion

Our main aim in the present study was to compare indices of endothelial function to investigate lethal and sublethal effects of oxygen product(s) on endothelium. We also wished to determine whether hydrogen peroxide or superoxide was the main product generated by xanthine oxidase that induced sublethal effects on endothelial cells. Four conventional indices of cytotoxicity (cell morphology, uptake of adenine, release of ^{51}Cr, and uptake of vital dye) were monitored and three parameters of cellular function were selected as possible indicators of sublethal damage: PG production, K^+ efflux (using ^{86}Rb), and release of cytoplasmic purines.

We confirmed previous reports of endothelial cytotoxicity induced by hydrogen peroxide: changes in morphology, release of ^{51}Cr, uptake of vital dye, and inhibition of adenine uptake were all observed with concentrations of $H_2O_2 >$ 300 μM. Cellular functions (PG production, K^+ efflux, and purine release) that had previously been shown to undergo physiological variations in situations where cell viability was unaffected (10, 15–18) were all affected by much lower concentrations of H_2O_2 than those which had lethal effects: threshold active concentrations were <10 μM for all three parameters. Similar effects on these parameters of endothelial cell function were also produced by xanthine plus xanthine oxidase, but experiments with SOD and catalase established that H_2O_2 was the agent responsible, rather than oxygen radicals. The concentrations of H_2O_2 generated by xanthine oxidase were not measured directly, but estimations based on cytochrome c reduction indicated that the maximum concentration of H_2O_2 produced was ~10 μM. However, the effects of enzymically generated H_2O_2 on K^+ efflux were greater than those achieved with reagent H_2O_2, even at 100 μM (see Figs. 1 and 2). This may reflect the delivery of H_2O_2 to the cells, which appears to be an important factor in determining how it is detoxified (19–21). Thus, nascent H_2O_2 (such as that produced by xanthine oxidase) is metabolized mainly by the glutathione cycle rather than by catalase, whereas reagent H_2O_2 is metabolized by both enzyme systems (21). Exposure to H_2O_2 not only stimulated PG production, K^+ efflux, and [^{3}H]purine release directly but also blocked subsequent responses of the cells to other stimuli. For example, agents such as ATP and bradykinin were incapable of stimulating the production of PG (PGI_2 and PGE_2) after the cells had been exposed to H_2O_2. This effect of H_2O_2 was apparent only at high oxygen tension and the concentration threshold was >100-fold lower than that for direct effects of H_2O_2 on endothelial cells.

Our results show, first, that the extent of endothelial cytotoxicity observed can depend on the method used to assess it. Endothelial morphology, ^{51}Cr release, vital dye uptake, and uptake of adenine showed similar sensitivity to H_2O_2 (i.e., all parameters were affected at the same concentration) but the extent of the changes observed in the different parameters varied widely. For example, at 1,000 μM H_2O_2, >90% of the cells took up vital dye and adenine uptake was

inhibited by >70%, but ^{51}Cr release was increased by only ~20%. Therefore, for experiments with endothelial cells in culture, we regard ^{51}Cr release as less suitable for monitoring cell damage than adenine uptake. Not only is the size of the response to a given damaging stimulus greater, but measurements of adenine uptake do not require prelabeling the cells (labeling with ^{51}Cr for subsequent release studies requires preincubation for several hours).

The mechanism(s) by which H_2O_2 affects endothelial cell functions remains to be determined. K^+ efflux and PG production, both of which are stimulated directly by low concentrations of H_2O_2, are known to be calcium dependent (8, 22) but experiments with the calcium ionophore A23187 demonstrated differences in the patterns of K^+ efflux and [^{3}H]purine release induced by H_2O_2 and by the ionophore. The stimulation of PG production by H_2O_2 could result from either the release of arachidonic acid, the activation of cyclo-oxygenase, or a combination of both. Taylor et al. (23) have shown that t-butyl hydroperoxide stimulates arachidonate release and cyclo-oxygenase activity in bovine pulmonary artery endothelial cells and that H_2O_2 has similar effects in human lung fibroblasts (24). The requirement of the continuous presence of peroxide for activation of purified cyclo-oxygenase has also been demonstrated (25, 26). The indirect effect of H_2O_2 at high O_2 tensions in inhibiting PG production could be due to the formation of an oxidizing species (by the action of peroxidases on hydroperoxides in the cell), which has been proposed to explain the observed inactivation of purified cyclo-oxygenase (25, 27). Egan et al. (28) have shown that such a species can be produced from H_2O_2. Hydroperoxy fatty acids have also been shown to inhibit cyclo-oxygenase activity (29). Both oxidizing species (30) and hydroperoxy fatty acids (31) can inhibit PGI_2 synthase without affecting thromboxane synthase and prostaglandin isomerase but we did not find selective inhibition of PGI_2 production over PGE_2 production after exposure to H_2O_2, suggesting that its effect was at the level of the cyclo-oxygenase. However, we have not yet investigated the possibility that there are effects of H_2O_2 (both indirect and direct) on the release of arachidonic acid.

More work is needed to determine the full spectrum of effects of H_2O_2 on endothelium and on other cell types. However, the present results do emphasize that to evaluate the effects of a potentially toxic agent, it is not sufficient to monitor one conventional index of cell damage such as ^{51}Cr release. Not only is this relatively insensitive (in that the responses measured are small, and since better indices of cell damage exist, e.g., adenine uptake), but important biological functions of the cell can be affected by concentrations of a toxic agent far below those that have lethal effects.

Summary

The responses of pig aortic endothelial cells to sublethal doses of potentially toxic stimuli were investigated by monitoring K^+ efflux, prostaglandin production, and the release of cytoplasmic purines. Xanthine plus xanthine oxidase reversibly stimulated these three parameters of endothelial cell function at doses that were not cytotoxic, as measured by chromium release, adenine uptake, and vital dye exclusion. The effects of xanthine plus xanthine oxidase were inhibited by catalase but not by superoxide dismutase, suggesting that H_2O_2 was respon-

sible. Reagent H_2O_2 also reversibly stimulated K^+ efflux, prostaglandin production, and the release of purines. The threshold concentration of H_2O_2 for these effects was ~10 μM, which was at least 30-fold lower than that which caused cytotoxicity.

In addition to the direct effect of H_2O_2 in stimulating prostaglandin production (PGI$_2$ and PGE$_2$), prior exposure of endothelial cells to lower doses of H_2O_2 (<0.1 μM) at high oxygen tension inhibited the subsequent stimulation of prostaglandin production by ATP, A23187, and H_2O_2 itself. We conclude that H_2O_2 has substantial effects on endothelial physiology at doses up to 3,000-fold lower than those which induce cytotoxicity.

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