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Research Article

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Neutrophil–Endothelial Cell Interaction

Evidence for and Mechanisms of the Self-Protection of Bovine Microvascular Endothelial Cells from Hydrogen Peroxide–induced Oxidative Stress

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Abstract

Bovine microvascular endothelial cells (MEC) were able to degrade the H_2O_2 generated by phorbol myristate acetate-activated bovine neutrophils or by glucose oxidase with a maximal capacity of 4.0±1.2 (SD) nmol/10^6 cells/min, corresponding to the H_2O_2 released by about 3×10^6 neutrophils. H_2O_2 degradation occurred via the glutathione redox cycle and catalase. Degradation via the glutathione redox cycle was coupled with a marked stimulation of the hexose monophosphate shunt activity. The effect of H_2O_2 on ethidium bromide exclusion and on succinate oxidation was studied. Neither parameter was altered when MEC were exposed to H_2O_2 produced at rates within their degradative capacity. As soon as this was exceeded, impairment of both functions occurred. It is concluded that endothelial cells can protect themselves from H_2O_2-induced injury in a well-defined range of environmental H_2O_2 concentrations by actively degrading the peroxide.

Introduction

Studies in vitro, with cultured endothelial cells, have shown that these cells are lethally damaged when exposed to activated neutrophils (PMN) (1–4). It has been emphasized that such damage results from the oxidative stress caused by the oxygen metabolites released by PMN (1–6), particularly H_2O_2 (4–6). Even in vivo, endothelial cells may be exposed to oxidants, for example, at sites of inflammation, where PMN are recruited and activated, or when PMN are activated intravascularly, as in the adult respiratory distress syndrome (ARDS) (7, 8). The question then arises as to whether endothelial cells inevitably undergo damage whenever they come into contact with activated PMN or whether they possess protective mechanisms against the oxidative stress. In this report we provide evidence that microvascular endothelial cells (MEC)[1] are able to degrade H_2O_2 and we describe their biochemical pathways for H_2O_2 degradation.

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1. *Abbreviations used in this paper:* BCNU, 1,3-*bis*(chloroethyl)-1-nitrosourea; GSH, reduced glutathione; GSSG, oxidized glutathione; HMPS, hexose monophosphate shunt; KRP, Krebs-Ringer phosphate (solution); MEC, microvascular endothelial cells; PMA, phorbol-12-myristate-13-acetate.

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Methods

Preparation of cells. MEC in high yield were isolated from bovine cavernous bodies, as previously described (9). Briefly, bovine cavernous bodies, freed from blood by perfusion with a solution of Krebs-Ringer phosphate (KRP) supplemented with 20% heat-inactivated (30 min at 56°C) bovine serum and 5.5 mM glucose through the cavernous bodies artery, were filled with KRP containing 150 U/ml collagenase and incubated for 20 min at 37°C. At the end of this period the collagenase-containing solution was drained and the cells were collected by centrifugation, washed, and resuspended at a concentration of $1–2.5 \times 10^8$/ml in KRP supplemented with 20% inactivated and dialyzed bovine serum, 2% bovine serum albumin (Fraction V), and 5.5 mM glucose. This procedure yields $2.5–4 \times 10^8$ cells per animal. Cell viability ranged from 96% to 98% as determined by the trypan blue dye exclusion test. Endothelial cells were identified by examination under the electron microscope and by immunofluorescence assays of factor VIII with rabbit antibovine factor VIII antibodies (97–99% positive).

Granulocytes were isolated from bovine blood according to Carlson and Kaneko (10), and suspended in Mg^{++}-free KRP at a concentration of 10^8 cells/ml. The preparations contained 95–98% neutrophils. More than 95% of the cells were viable as judged by the trypan blue exclusion test.

Enzyme assays. Performed on total cell homogenate, these assays used aliquots of the suspension of freshly isolated MEC or PMN that were washed twice and resuspended in ice-cold KRP. Homogenization was then performed as previously described for polymorphonuclear leukocytes (11). The homogenate was diluted in KRP and immediately assayed for enzymatic activities at 37°C. Aliquots were stored at −20°C for subsequent protein determination. This was performed by the Lowry method (12), using crystalline bovine serum albumin as standard. Catalase activity and guaiacol peroxidase activity were measured as previously described (13, 14). Glutathione peroxidase and glutathione reductase were measured according to the method of Reed (15), as modified by Gennaro et al. (16). To test the influence of 1,3-*bis*(chloroethyl)-1-nitrosourea (BCNU) on glutathione reductase activity, 10^7 MEC were incubated for 10 min at 37°C in 1 ml KRP containing 5.5 mM glucose and 100 μg/ml BCNU or 0.1% ethanol control. The cells were then washed and homogenized as described above and immediately assayed for glutathione reductase activity.

H_2O_2 assay. Hydrogen peroxide was measured fluorimetrically by a previously described method (17). The method is based on the conversion of the nonfluorescent compound homovanillic acid to the highly fluorescent 2,2′-dihydroxy-3-3′-dimethoxy-biphenyl-5-5′-diacetic acid (excitation 315 nm, emission 425 nm) by horseradish peroxidase in the presence of H_2O_2. Reference standards of known H_2O_2 concentrations were obtained by diluting reagent grade H_2O_2. H_2O_2 concentration was assayed spectrophotometrically using an extinction coefficient of 62.2 cm^{-1} M^{-1} at 230 nm (18).

Metabolic assays. H_2O_2 degradation by MEC was tested by incubating the cells with glucose oxidase or phorbol myristate acetate (PMA)-activated granulocytes at 37°C in 1 ml KRP containing 5.5 mM glucose under continuous shaking. At the end of the incubation, 25–100 μl of the incubation mixtures were transferred into a fluorimeter cuvette containing 1.9 ml of KRP, 40 μg of horseradish peroxidase, and 20 μM homovanillic acid for the H_2O_2 assay.

Oxygen uptake was monitored by a Clark oxygen electrode (Yellow

Springs Instrument Co., Yellow Springs, OH) at 37°C as previously described (19). PMA-activated granulocytes or glucose oxidase were incubated in KRP containing 5.5 mM glucose and in the presence of either endothelial cells or an equivalent amount of endothelial cells suspending medium. The assay volume was 2 ml. The mixture was stirred throughout the assay.

Production of $^{14}CO_2$ by MEC from differentially labeled [^{14}C]glucose was measured as previously described (20). Incubations were performed in 25-ml Erlenmeyer flasks with a center well. MEC (10^7) were placed in the main compartment of the flask, in 1 ml of KRP containing 5.5 mM glucose and 2 μCi of the radioactive substrate. The center well contained 0.25 ml 20% KOH. The flasks were sealed with rubber caps and incubated at 37°C in a Dubnoff shaker (Valentini Orlando, Milan, Italy). The reactions were stopped by the addition of trichloroacetic acid, final concentration 5%, into the main compartment through the rubber cap. After standing for 30 min at 37°C and for additional 15 min at 4°C the contents of the center wells were transferred into liquid scintillation vials containing 4 ml of methanol, 6 ml of toluene, and 55 mg of permablend III for counting in a Beckman LS-100 spectrometer (Beckman Instruments, Inc., Palo Alto, CA).

Production of labeled and unlabeled lactate by MEC was measured on the trichloroacetic acid extracts of the main compartments of the flasks, as previously described (20). Enzymatic estimation of lactate was performed directly on the acid extracts, according to the method of Hohorst (21). The samples were then further extracted as described by Barker and Summerson (22) and the radioactive lactate present in the extracts was measured by liquid scintillation counting, as described above for the $^{14}CO_2$.

To determine the incorporation of [U-^{14}C]glucose into the lipid fraction incubations were carried out as described for the $^{14}CO_2$ production, with the exception that the reactions were stopped by freezing at −20°C instead by addition of trichloroacetic acid. The contents of the main compartments were used for lipid extraction according to the method of Folch et al. (23). The radioactivity in the lipid extracts was counted using the same method described for the $^{14}CO_2$.

Glucose uptake was measured by the procedure of McCall et al. (24), modified as follows. MEC (10^7) were incubated in 1 ml of KRP containing 5.5 mM glucose at 37°C under shaking. The reaction was initiated by the addition of 0.5 μCi of 2-[1-^{3}H]-deoxyglucose and stopped after 30 min as described by Naccache et al. (25). The incubation mixtures were layered over 0.4 ml of AR 200 silicon oil (density 1.04 g/ml) in 1.5-ml plastic tubes which were then centrifuged for 0.5 min at 8,000 rpm in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). The suspending medium, which remained above the oil, and the oil layer were aspirated by suction and the cell pellet was lysed with 10 μl of 0.5% Triton X-100. The bottom of tubes containing the cell pellets was then excised and transferred into vials containing 15 ml of the same scintillation cocktail employed for $^{14}CO_2$ counting. Radioactivity was then counted as described for $^{14}CO_2$. No radioactivity was found in the oil layer. The radioactivity taken up by the cells during the 30-min incubation was corrected for the radioactivity of time zero (samples centrifuged immediately after the addition of [^{3}H]deoxyglucose).

Estimation of the total glutathione content (GSH + GSSG) and of oxidized glutathione (GSSG) was performed on the content of the main compartment of the Erlenmeyer flasks used to determine the $^{14}CO_2$ production from labeled glucose. For the assay of GSH + GSSG, the reaction was stopped by addition of 150 μl of trichloroacetic acid (final concentration 5%), as described for $^{14}CO_2$ production, while for the assay of GSSG, 75 μl of a mixture of 11.5 mM tetrasodium ethylenediaminetetracetate and 0.23 mM *N*-ethylmaleimide were added, in order to remove the GSH present (26), 20 s before a 75-μl trichloroacetic acid addition. The content of the main compartments was then centrifuged at 10,000 *g* for 5 min and extracted 15 times with ether (26). Total glutathione (GSH + GSSG) was then measured by the so-called "cyclic method" described by Owens and Belcher (27), where glutathione is oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and the product is reduced by NADPH in the presence of glutathione reductase. The NADPH decrease was then measured fluorimetrically, essentially as described by Voetman et al. (28). In particular, 50–250 μl of the extract (containing an average of 0.5 nmol GSH) were incubated at 37°C in sodium phosphate buffer, pH 7.4 (10 mM), with tetrasodium ethylendiamine tetracetate (1 mM), 5,5'-dithiobis-(2-nitrobenzoic acid) (15 nmol), and NADPH (5 nmol) in a final volume of 1.2 ml. The cyclic reaction was started by adding 1 μg of glutathione reductase, and the decrease in fluorescence of NADPH was recorded (excitation 360 nm, emission 450 nm). The glutathione concentration in the samples was then calculated by comparison with a set of GSSG and GSH standards of known concentration which were subjected to the same extraction procedure with ether described above for the cell samples. The results were expressed as "GSSG equivalents," where 1 nmol GSSG equivalent = 2 nmol GSH.

Assessment of cell damage. Two assays were used: combined uptake of ethidium bromide and fluorescein diacetate and oxidation of [1,4-^{14}C]succinic acid.

The ethidium bromide and fluorescein diacetate uptake is based on the principle that intact cells exclude ethidium bromide but take up fluorescein diacetate which is hydrolized inside the cell into fluorescein and acetate. Hence, viable cells display a bright-green fluorescence when excited at 488 nm, whereas dead cells stain red owing to coupling of ethidium bromide with DNA (29). At the required time, the rest of the incubation mixtures used to assay H_2O_2 degradation by MEC, as described above, was centrifuged at 200 *g* for 5 min. A part of the samples was then resuspended in KRP containing 5.5 mM glucose, 25 μg/ml fluorescein diacetate, 0.5 mg/ml ethidium bromide, and 20 μg/ml catalase. After 5 min of incubation at 37°C and two washes in KRP, the number of red and green cells was counted under the fluorescence microscope. The other part of the samples was resuspended in KRP containing 5.5 mM glucose and 20 μg of catalase, postincubated for 3 h, and then centrifuged and treated with ethidium bromide and fluorescein diacetate as described above. When assays were performed on MEC incubated together with neutrophils, the two cell types were easily recognized from each other. In fact, all the nuclei of PMA-activated neutrophils stained with ethidium bromide and were therefore easily distinguishable from the differently shaped and sized ethidium bromide-positive nuclei of endothelial cells and from fluorescein-positive endothelial cells.

Oxidation of [1,4-^{14}C]succinic acid was measured as an index of mitochondrial function. MEC (10^7) were incubated for 30 min at 37°C in 10 ml of KRP containing 5.5 mM glucose and varying amounts of glucose oxidase. After centrifugation at 200 *g* for 5 min they were resuspended in 1.5 ml of KRP containing 5.5 mM glucose, 20% dialyzed serum, 2% bovine albumin, and 30 μg of catalase. The mixture was then transferred into the main compartment of Erlenmeyer flasks. An additional 30-min incubation at 37°C was started by addition of 1 mM [1,4-^{14}C]succinic acid (initial specific activity, 1 μCi/μmol). The reaction was then stopped by addition of trichloroacetic acid, 5% final concentration, and the $^{14}CO_2$ production was then measured as described above.

Reagents. Horseradish peroxidase (330 U/mg), glucose oxidase type VII (220 U/mg), bovine superoxide dismutase (2,800 U/mg), homovanillic acid, phorbol-12-myristate-13-acetate (PMA), ethidium bromide, fluorescein diacetate, trypan blue, and *N*-ethylmaleimide were purchased from Sigma Chemical Co., St. Louis, MO. PMA stock solutions (2 mg/ml) were stored in dimethylsulfoxide at −70°C. BCNU (Nitrumon) was obtained from Sintesa S.A., Brussels, and dissolved in ethanol at a 100 mg/ml stock just before use. Permablend III was from Packard Instrument Co., Inc., Downers Grove, IL. Hydrogen peroxide, dimethylsulfoxide, tetrasodiumethylenediaminetetracetate, and trichloroacetic acid were from Merck, Darmstadt, Federal Republic of Germany. 5,5'-dithiobis-(2-nitrobenzoic acid) and silicone oil AR 200 were from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany. Catalase (65,000 U/mg), NADPH, oxidized glutathione, reduced glutathione, and glutathione reductase (1 mg/ml) were from Boehringer Mannheim, Federal Republic of Germany. Collagenase CLS II (150 U/mg) was from Worthington Diagnostic Systems, Freehold, NJ. Triton X-100 was from BDH Chemicals, Poole, United Kingdom. Bovine serum albumin was from Miles Laboratories Inc., Goodwood, South Africa. Rabbit antibodies against bovine factor VIII were a generous gift from Dr. E. P. Kirby, Temple University, Philadelphia, PA. Goat antiserum against rabbit

Table I. Activities of H_2O_2-degrading Enzymes in Bovine Microvascular Endothelial Cells and Bovine Blood Neutrophils

	Endothelial cells	Neutrophils
Catalase (*μmol H_2O_2/min*)	3.00±0.57 (7)	0.27±0.02 (4)
Glutathione peroxidase (*nmol NADPH/min*)	2.47±0.12 (7)	1.63±0.25 (5)
Glutathione reductase (*nmol NADPH/min*)	2.98±0.14 (7)	5.16±0.24 (5)
Peroxidase (*nmol tetraguaiacol formed/min*)	0.1 (5)	44.1±8.6 (5)
Protein (*μg*)	104.1±5.0 (17)	91.0±4.8 (6)

Values are derived from 10^6 cells. The means of (*n*) experiments±1 SE are reported.

gammaglobulin was from Behring Institute, Marburg, Federal Republic of Germany. ^3H- and ^{14}C-labeled substrates were obtained from the Radiochemical Center, Amersham, United Kingdom. The Krebs-Ringer phosphate (KRP) solution used had the following composition: 0.154 M NaCl, 0.154 M KCl, 0.154 M $MgCl_2$, 0.1 M sodium phosphate buffer (pH 7.4) in the following ratios 100:4:1:21 (vol/vol).

Statistical analyses. The results in Fig. 2 are described with curves representing multiple linear regression quadratic equations ± mean square deviations, as computed according to the Gauss-Jordan method (30, 31).

Values in figures and text are given as the means±SE unless otherwise stated. In cell viability assays (Figs. 3–5) the significance of the differences between the mean values of treated cells and those of control cells was calculated with the paired Student's *t* test (two tailed) by using a Hewlett-Packard model 25 programmable calculator (Hewlett-Packard Co., Palo Alto, CA).

Results

H_2O_2-degrading enzymes of endothelial cells. As shown in Table I, MEC have catalase, glutathione peroxidase, and glutathione reductase activities but no peroxidase activity, as assayed by the guaiacol method. Activities of the same enzymes in neutrophilic leukocytes, a cell type that produces and metabolizes H_2O_2, are reported in Table I for comparison. The high catalase activity of MEC in comparison to that of neutrophils is remarkable and suggests that this enzyme may play a significant role in protecting endothelial cells from oxidative stress.

H_2O_2 degradation by endothelial cells. MEC were incubated with two H_2O_2 sources, that is, PMA-stimulated PMN and the cell-free system glucose–glucose oxidase, and the H_2O_2 that accumulated in the medium was measured. The experiments were performed both in the presence and absence of the inhibitor sodium azide (NaN_3) to test the contribution of catalase, which is an NaN_3-sensitive enzyme, to H_2O_2 degradation by MEC. Preliminary experiments were performed to establish the kinetics of H_2O_2 production by glucose oxidase and PMA-activated PMN. As shown in Fig. 1, H_2O_2 production by glucose oxidase was linear for at least 2 h. It was unaffected by NaN_3 (not shown). With PMA-stimulated PMN, the H_2O_2 production rate was linear for 10–12 min and then progressively declined. NaN_3 did not appreciably affect the H_2O_2 production by stimulated PMN during the first 10–12 min, but afterwards the amount of H_2O_2 that accumulated in the medium was higher in the presence than in the absence of the inhibitor. On the basis of these results a 10-min period of exposure of MEC to the H_2O_2-generating systems was selected. The appropriate controls were glucose–glucose oxidase or activated PMN incubated without MEC. With either H_2O_2-generating system, less H_2O_2 was recovered in the presence of MEC than in their absence, suggesting that H_2O_2 degradation by MEC had taken place. As shown in Fig. 2, the total amount of H_2O_2 degraded by MEC increased as a function of H_2O_2 production, until a plateau was reached at a level of 4.1±1.2 (SD) nmol H_2O_2 degraded per 10^6 cells/min. Because the average H_2O_2 production rate by the stimulated neutrophils in the initial linear phase was 1.3±0.5 (SD) nmol per 10^6 PMN/min, six experiments (in the presence of NaN_3 it was 1.5±0.8 [SD] nmol per 10^6 PMN/min, six experiments), it was calculated that a single endothelial cell could degrade the H_2O_2 released by about three neutrophils. NaN_3 strongly inhibited, although it did not abolish, H_2O_2 degradation by MEC, suggesting an involvement of catalase. Control experiments showed that (*a*) MEC did not produce H_2O_2 and (*b*) that dialyzed bovine serum and albumin, which were present in the suspension medium of the endothelial cells, did not affect the recovery of H_2O_2 at the concentrations present in the assays (0.5% and 0.05%, respectively). Control experiments were also run to rule out the possibility that the diminished recovery of H_2O_2 in the presence of MEC could be accounted for by the inhibition of PMN activation or glucose oxidase activity by MEC. This was tested by measuring the effect of MEC on the oxygen consumption by PMA-stimulated PMN and glucose oxidase. The results are shown in Table

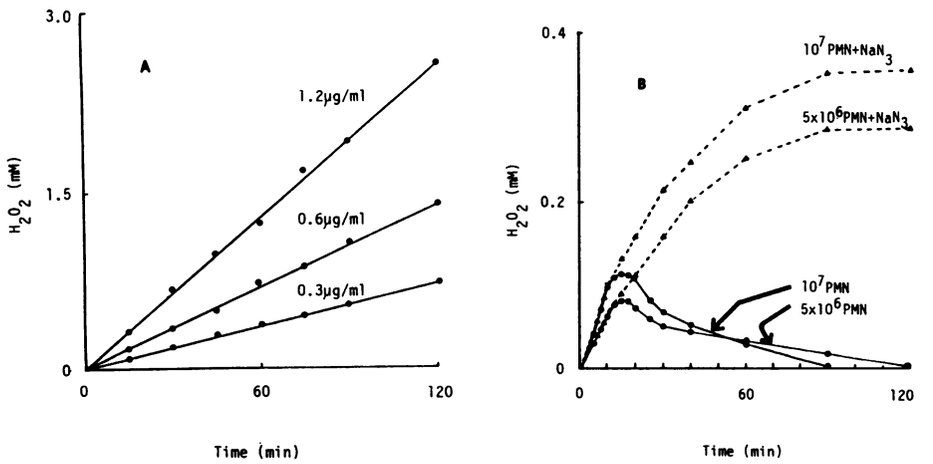

Figure 1. Time course of H_2O_2 production by glucose-glucose oxidase (*A*) or by activated PMN (*B*). Glucose oxidase or PMA (0.1 μg/ml)-stimulated PMN were incubated at 37° in 1 ml of KRP containing 5.5 mM glucose, and the H_2O_2 generated was measured at various intervals. The concentrations of glucose oxidase and the number of PMN used are indicated in the figure. Values are single measures from one representative experiment. (●) No NaN_3; (▲) with NaN_3 (2 mM).

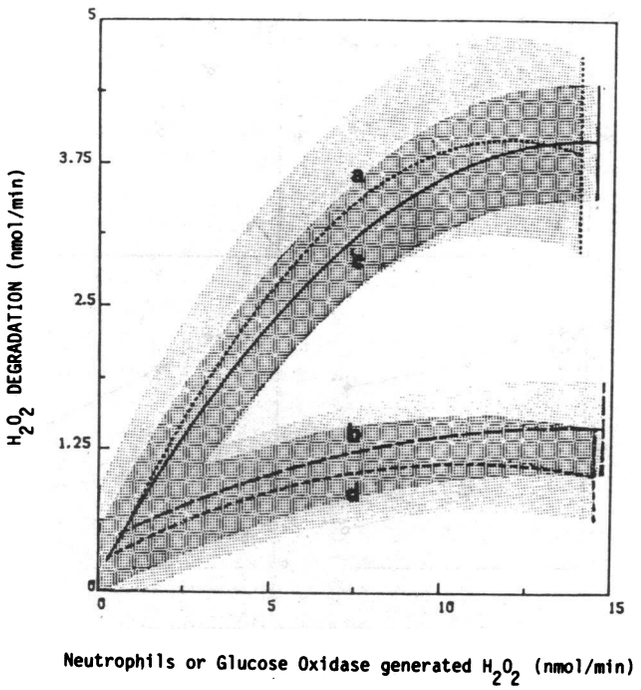

Figure 2. Degradation by endothelial cells of H_2O_2 produced by activated neutrophils or by glucose-glucose oxidase. Endothelial cells (10^6) were incubated for 10 min with varying amounts of glucose oxidase (*a*), glucose oxidase and NaN_3 (2 mM) (*b*), or with different numbers of PMA (0.1 μg/ml)-stimulated neutrophils (0.25–10 × 10^6 cells) (*c*) or PMA-stimulated neutrophils and NaN_3 (2 mM) (*d*). The values of H_2O_2 degraded by endothelial cells were calculated from the difference of H_2O_2 measured in samples without and with endothelial cells. Curves represent multiple linear regression quadratic equations ± mean square deviations, as computed on 40–50 values from six experiments.

II. The data show unequivocably that, in the presence of NaN_3, MEC did not affect oxygen uptake by either stimulated PMN or glucose oxidase. The data obtained in the absence of NaN_3 require a more detailed explanation. In the presence of MEC, the oxygen uptake by stimulated PMN and glucose oxidase was 24 and 20 nmol, respectively, lower than the sum of the oxygen taken up by MEC and that by stimulated PMN or glucose oxidase, respectively. These amounts of oxygen are considerably lower than the amounts of H_2O_2 degraded in the same conditions, indicating at first sight that at least most of the disappearance of the hydrogen peroxide cannot be accounted for by inhibition of PMN activation or glucose oxidase activity. In addition, it is easily demonstrable that the difference in oxygen uptake observed in the presence of MEC is not due to inhibition, but is accounted for by the oxygen given back by the aliquot of H_2O_2 degraded by catalase. In fact, this aliquot of H_2O_2, as judged from a comparison of H_2O_2 degradation in the presence and in the absence of NaN_3, amounts to 45 and 54 nmol in the experiments with glucose oxidase and with PMN, respectively (this calculation is based on the assumption that the amount of H_2O_2 degraded through the NaN_3-insensitive pathway is not increased in the absence of NaN_3. This is indeed the case as it will be shown in the paragraph on the glutathione cycle activity). Degradation of these amounts of H_2O_2 by catalase is expected to give back 27 and 22.7 nmol oxygen, respectively, which strictly matches the decreased oxygen uptake, in the absence of azide, of 24 and 20 nmol oxygen by stimulated PMN and glucose oxidase, respectively. In conclusion, it is clear that endothelial cells do not appreciably inhibit glucose oxidase activity or PMN activation in our experimental conditions.

Viability of endothelial cells exposed to H_2O_2. Two functions were monitored, that is, succinic acid oxidation, as an index of mitochondrial function, and exclusion of the vital dye ethidium bromide. No statistically significant impairment of succinic acid oxidation was observed in MEC incubated for 30 min with glucose oxidase until the rate of H_2O_2 production reached 5 nmol/min per 10^6 cells. From this concentration onward, succinic acid oxidation was impaired (Fig. 3). Comparable results were obtained with ethidium bromide (Fig. 4). In fact, after 30 min of incubation there was no statistically significant impairment of the dye excluding function until the H_2O_2-generation rate reached 5 nmol/min per 10^6 cells. Postincubation for 3 h of MEC incubated for 30 min with glucose oxidase did not appreciably increase the number of MEC taking up ethidium bromide in samples exposed to H_2O_2 produced at rates below 5 nmol/min, suggesting that the maintenance of the ethidium bromide excluding function in these samples reflects cell integrity rather than a latent lesion which needs longer time to become manifest. Under the same experimental conditions, the widely used vital dye trypan blue did not reveal any cell damage even at H_2O_2 generation rates of 10 nmol/min (five experiments, results not shown), indicating that ethidium bromide uptake is an earlier

Table II. Effect of Endothelial Cells on the Oxygen Uptake and H_2O_2 Production by PMA-stimulated PMN and by Glucose-Glucose Oxidase

	Oxygen uptake		H_2O_2 production	
	−NaN_3	+NaN_3	−NaN_3	+NaN_3
	nmol/10 min	*nmol/10 min*	*nmol/10 min*	*nmol/10 min*
PMA-stimulated PMN	348±8	357±11	154±6	180±7
+ endothelial cells	340±9	360±14	55±3	135±8
Glucose-glucose oxidase	150±5	152±4	136±4	151±3
+ endothelial cells	146±6	150±5	48±6	108±3
Endothelial cells	16±2	undetectable	undetectable	undetectable

PMA (0.1 μg/ml)-stimulated PMN (10^7) or glucose oxidase (0.8 μg) were incubated for 10 min in the absence and presence of endothelial cells (10^7) and in the absence and presence of NaN_3 (2 mM). Values (nmol/10 min) are means±1 SD of triplicate assays from a typical experiment.

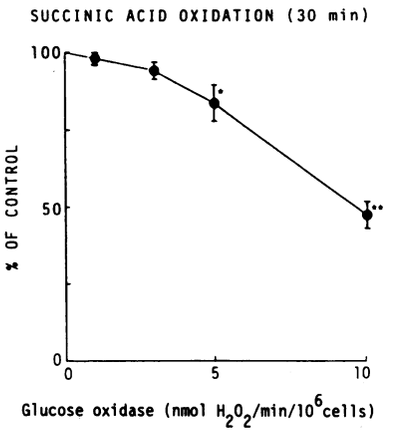

Figure 3. Effect of H_2O_2 produced by glucose-glucose oxidase on succinic acid oxidation by endothelial cells. 10^7 endothelial cells were incubated for 30 min at 37°C in KRP containing 5.5 mM glucose and varying amounts of glucose oxidase, then centrifuged, resuspended in KRP containing 5.5 mM glucose, 1 mM [1,4-^{14}C]succinic acid, and 20 μg/ml catalase, and incubated again for 30 min. The reaction was then stopped by addition of 5% trichloroacetic acid (final concentration, wt/vol) for the estimation of the $^{14}CO_2$ formed. Oxidation of succinic acid was calculated on the basis of an initial specific activity of [1,4-^{14}C]succinic acid of 1,700 cpm/nmol. Results (means±1 SE of six experiments) are given as percentage of control values, that is, values of samples incubated without glucose oxidase. Control values were 0.82±0.21 nmol succinic acid per 10^7 cells/30 min. Statistical significance of differences from control values (paired *t* test): $*P < 0.05$; $**P < 0.001$. Other results were not significantly different from control values.

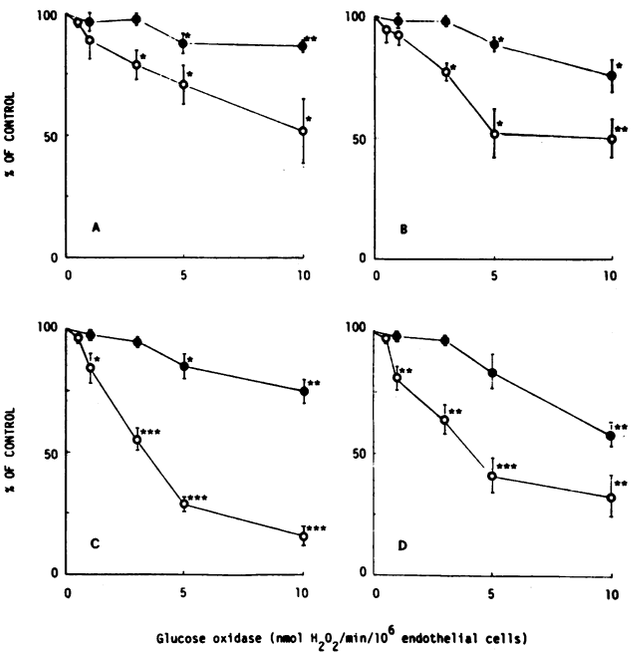

Figure 4. Effect of H_2O_2 produced by glucose-glucose oxidase on ethidium bromide exclusion by endothelial cells. (*A*) 30-min exposure to H_2O_2; (*B*) 30-min exposure to H_2O_2 followed by 3-h postincubation in the absence of H_2O_2; (*C*) 2-h exposure to H_2O_2; (*D*) 2-h exposure to H_2O_2 followed by 3-h postincubation in the absence of H_2O_2. The results (means±1 SE of five experiments) are reported as percentage of control values, that is, values of samples incubated without glucose oxidase. The percentage of cells excluding ethidium bromide in control samples ±NaN_3 was (*A*) 87±2 SE, (*B*) 83±5 SE, (*C*) 84±1 SE, and (*D*) 84±2 SE. Statistical significance of differences from control values (paired *t* test): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Other results were not significantly different from control values. (●) No NaN_3; (2 m*M*) with NaN_3 (○).

index of cell damage as compared to trypan blue uptake. Even a longer exposure (2 h) of MEC to glucose oxidase did not result in statistically significant increase of ethidium bromide uptake until the rate of H_2O_2 production reached 5 nmol/min. Again, postincubation for 3 h of MEC incubated for 2 h with glucose oxidase did not appreciably change the pattern, except for cells exposed to H_2O_2 produced at rates of 10 nmol/min.

When the experiments were performed in the presence of NaN_3, which inhibited most of the degradative capacity of the cells, the viability was impaired even at H_2O_2 concentrations that had no effect in the absence of the inhibitor. Experiments with NaN_3 could not be done with succinate, because sodium azide inhibits mitochondrial oxidation. In control experiments with heat-inactivated glucose oxidase or with catalase (20 μg/ml) included in the incubation medium, no impairment of ethidium bromide exclusion or succinic acid oxidation was observed.

The same type of experiment was carried out with MEC exposed to H_2O_2 produced by PMA stimulated PMN (Fig. 5). 10^6 MEC were incubated with a number of PMN varying from 0.5 to 10 × 10^6 cells (equivalent to an H_2O_2 production rate of 0.5–15 nmol/min). There was no ethidium bromide uptake by MEC after 30 min of incubation at PMN/MEC ratios up to 4:1. Only at a ratio of 10:1, a small number of stained cells began to appear. After 5 h of incubation, the number of ethidium bromide-positive cells was quite small at PMN/MEC ratios between 2:1 and 4:1 (corresponding to H_2O_2-generation rates between 3 and 6 nmol/min).

In the presence of NaN_3, the impairment of ethidium bromide exclusion was magnified after 5 h of incubation and became apparent also after 30 min of incubation. Addition of catalytically active catalase (20 μg/ml) to the incubation medium of endothelial cells with neutrophils prevented cell damage, whereas addition of heat-inactivated catalase or catalytically active superoxide dismutase (40 μg/ml) were ineffective in this respect, indicating that, even with PMN, H_2O_2 or an H_2O_2-derived product was the actual mediator of endothelial damage.

Taken altogether, the results reported in this paragraph indicate that, in our experimental set, endothelial cells can successfully deal, without any detectable damage, with H_2O_2 concentrations afforded by a continuously H_2O_2-generating source at rates up to 3–4 nmol/min per 10^6 cells, which corresponds to the maximum H_2O_2-degradative capacity of the cells, as shown in Fig. 1. Instead, when endothelial cell catalase is inhibited, cell damage occurs at much lower H_2O_2 concentrations.

Role of glutathione redox cycle and catalase in H_2O_2 degradation. In other cell types, for example, neutrophilic leukocytes, the activity of glutathione peroxidase is linked with the HMPS activity through the following mechanism: in the presence of H_2O_2, glutathione peroxidase oxidizes glutathione to GSSG, which is then reduced to GSH by glutathione reductase. This causes oxidation of NADPH to $NADP^+$ which activates the hexose monophosphate shunt (HMPS) (15). Therefore, when this sequence of reactions is operative, it is possible to measure the degradation of H_2O_2 through the glutathione peroxidase-reduc-

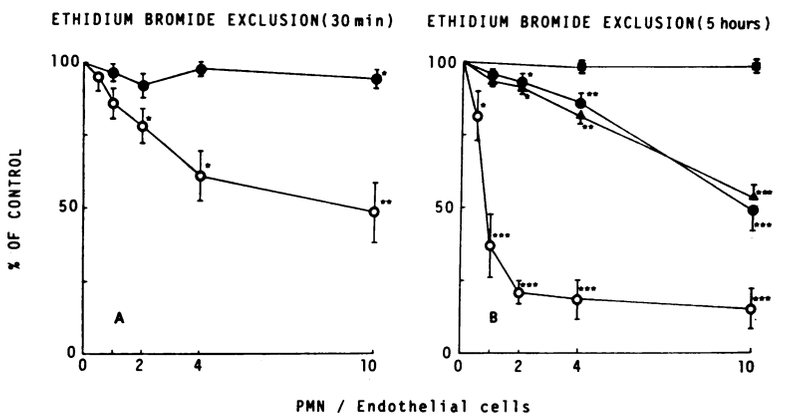

Figure 5. Effect of H_2O_2 generated by PMA-stimulated neutrophils on ethidium bromide exclusion by endothelial cells. 10^6 endothelial cells were incubated for 30 min (*A*) or 5 h (*B*) with different numbers of neutrophils and (●) in the absence or (○) presence of 2 mM NaN_3, (▲) 40 μg/ml superoxide dismutase, or (■) 20 μg/ml catalase. The results are expressed as percentage of control values, that is, values of samples incubated in the absence of neutrophils. The percentage of cells excluding ethidium bromide in control samples was 90±2 SE and 85±5 SE after 30 min and 5 h of incubation ±NaN_3, respectively. Values are means±1 SE of six experiments at 30 min and nine experiments at 5 h. Statistical significance of differences from control values (paired *t* test): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; other results were not significantly different from control values.

tase system by determining the activity of the HMPS. In the HMPS, CO_2 is generated from oxidation of glucose carbon 1. The resulting pentose molecule may be converted back into glucose with carbon 2 taking the carbon 1 position, which can then enter a second cycle (32). Hence assay of $^{14}CO_2$ production from both [1-^{14}C]glucose and [2-^{14}C]glucose gives a measure of the HMPS activity. Using this procedure we have obtained evidence that the HMPS is operative in endothelial cells and is stimulated by H_2O_2. Fig. 6 shows that untreated endothelial cells produced a small amount of $^{14}CO_2$ from [1-^{14}C]glucose, which increased by more than 25-fold after treatment with H_2O_2. The amount of $^{14}CO_2$ produced from [2-^{14}C]glucose was also small and sharply increased upon treatment with H_2O_2, indicating a certain degree of glucose recycling. The increase of [1-^{14}C]- and [2-^{14}C]glucose oxidation was not accompanied by an increased glucose uptake by the cells, as measured by [^{3}H]deoxyglucose uptake (24), nor by any significant increase of glucose oxidation through the tricarboxylic acid cycle, as measured by $^{14}CO_2$ production from [6-^{14}C]glucose, nor by increased lipid synthesis as measured by [U-^{14}C] incorporation into total lipids. These results indicate that the increased $^{14}CO_2$ production from [1-^{14}C]glucose and from [2-^{14}C]glucose reflects a specific stimulation of the HMPS by H_2O_2 rather than an overall stimulation of the glucose metabolism.

The HMPS stimulation was dose-dependent (Fig. 7) until the maximum HMPS potential was approached, as indicated by the stimulation obtained with methylene blue, a redox compound which increases the ratio $NADP^+$/NADPH by oxidizing NADPH, and hence directly activates the HMPS (33, 34). In

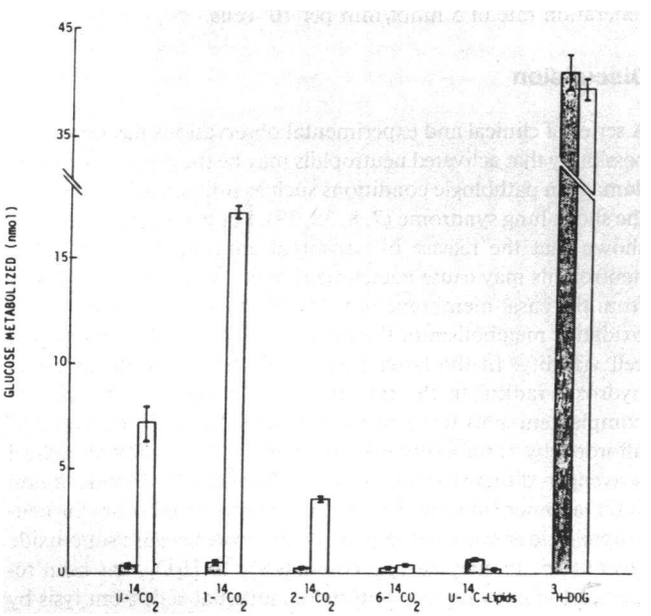

Figure 6. Effect of glucose-glucose oxidase-generated H_2O_2 on $^{14}CO_2$ production from [U-^{14}C]-, [1-^{14}C]-, [2-^{14}C]-, and [6-^{14}C]glucose on [U-^{14}C]glucose incorporation into lipids, and on 2-[1-^{3}H]-deoxyglucose (^{3}H-DOG) uptake by endothelial cells. 10^7 cells were incubated for 30 min in 1 ml of KRP containing 5.5 mM glucose and in the absence (*shaded bars*) or presence (*open bars*) of glucose oxidase producing H_2O_2 at a rate of 30 nmol/min. The amount of glucose metabolized was calculated on the basis of an initial specific activity of 650 cpm/nmol for [^{14}C]glucose and 65 cpm/nmol for [^{3}H]glucose. Means±1 SD of triplicate assays from one representative experiment.

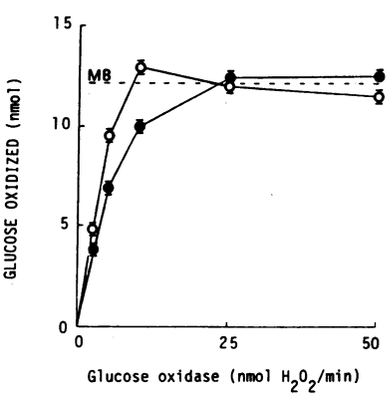

Figure 7. Effect of H_2O_2 generated by glucose-glucose oxidase and of methylene blue on [1-^{14}C]- plus [2-^{14}C]glucose oxidation by endothelial cells. 10^7 cells were incubated for 10 min with 2 μmol methylene blue (*MB*) or with varying amounts of glucose oxidase in the absence (●) and presence (○) of NaN_3 (2 mM), in 1 ml of KRP containing 5.5 μmol glucose and 2 μCi of either [1-^{14}C]- or [2-^{14}C]glucose. The amount of glucose oxidized was calculated from the sum of the $^{14}CO_2$ produced by parallel samples incubated with [1-^{14}C]- and with [2-^{14}C]glucose, on the basis of an initial specific activity for [^{14}C]glucose of 650 cpm/nmol. Glucose oxidation by cells incubated with methylene blue was 12.1±0.5 SD nmol, as indicated by the *dotted line.* Values are means±1 SD of triplicate assays from one representative experiment.

the presence of NaN_3, the stimulation of the HMPS was slightly higher than in the absence of the inhibitor at H_2O_2 concentrations, which gave a stimulation below the HMPS maximum potential. This is attributable to diversion of some H_2O_2 from degradation by catalase to degradation through the glutathione peroxidase-reductase system coupled with the HMPS. Activation of the HMPS by H_2O_2 was linear with time for at least 90 min (Fig. 8). The glutathione reductase inhibitor BCNU (35, 36), which in our conditions inhibited glutathione reductase of the endothelial cells by 89±4% (mean±SD, three experiments), prevented HMPS stimulation by H_2O_2 while it had no effect on glucose oxidase activity and on the HMPS stimulation by methylene blue (Fig. 9), indicating that the HMPS stimulation is coupled with H_2O_2 degradation through the glutathione redox cycle. Such a coupling is further supported by the data shown in Table III, that is (*a*) a ratio of 2:1 (line 9) was actually found between the H_2O_2 degraded (line 4, difference between the total amount of H_2O_2 degraded and the amount of glutathione oxidized) and the CO_2 generated by the cells (line 8), as theoretically expected if all the H_2O_2 degraded went through the glutathione cycle coupled with the HMPS (15) and (*b*) the concentration of oxidized glutathione (line 3) did not change when the cells, exposed in the presence of NaN_3 to increasing concentrations of H_2O_2 (line 1), degraded >90% of the environmental H_2O_2 (line 2), but began to increase as soon as the H_2O_2 production rates exceeded the degradative capacity of the cells. The 2:1 ratio between the total H_2O_2 degraded and the CO_2 formed in the presence of NaN_3 lends support for the additional conclusion that, when catalase is inhibited, the only H_2O_2-degrading pathway is the glutathione redox cycle coupled with the HMPS.

Having established that catalase and the glutathione cycle are the only pathways of H_2O_2 degradation in MEC, it is possible to calculate their relative contribution at different concentrations of environmental H_2O_2, even in the absence of inhibitors of the two pathways. In fact, the difference between the total amount of H_2O_2 degraded, directly estimated as in Fig. 1, and the amount of H_2O_2 degraded by the glutathione cycle, estimated as CO_2 production through the HMPS, gives the amount of the H_2O_2 degraded by catalase. Fig. 10 shows that at H_2O_2-generation rates that are below the maximal degradative potential of the glutathione cycle, 70% of the H_2O_2 was degraded through this pathway. At higher H_2O_2 concentrations, which exceeded the glutathione cycle potential, the relative contribution of catalase progressively increased and eventually became prominent. For example, 90% of the H_2O_2 was degraded by catalase at a H_2O_2 generation rate of 5 nmol/min per 10^6 cells.

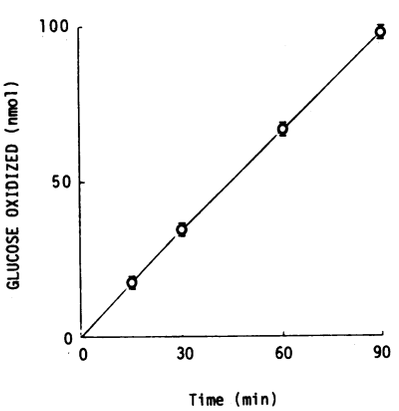

Figure 8. Time course of [1-^{14}C]- plus [2-^{14}C]glucose oxidation by endothelial cells exposed to H_2O_2 generated by glucose-glucose oxidase. 10^7 cells were incubated with glucose oxidase generating H_2O_2 at a rate of 4±0.3 nmol/min in 1 ml of KRP containing NaN_3 (2 mM), 5.5 μmol glucose, and 2 μCi of either [1-^{14}C]- or [2-^{14}C]glucose. The reactions were stopped by addition of 5% trichloroacetic acid (final concentration, wt/vol) at various intervals for the estimation of the $^{14}CO_2$ formed. The amount of oxidized glucose was calculated as in Fig. 7. Values are means±1 SD of triplicate assays from one representative experiment.

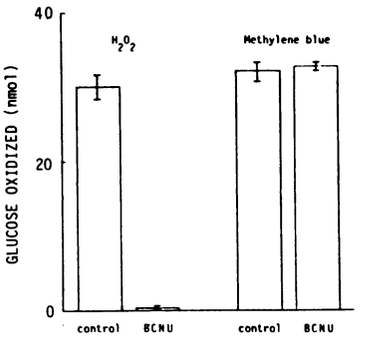

Figure 9. Effect of BCNU on [1-^{14}C]glucose oxidation by endothelial cells exposed to H_2O_2 generated by glucose-glucose oxidase or treated with methylene blue. 10^7 cells were preincubated for 10 min with 100 μg/ml BCNU or 0.1% ethanol control in 1 ml of KRP containing 5.5 mM glucose and 2 mM NaN_3. Then 2 μCi of [1-^{14}C]glucose and either 2 μmol methylene blue or glucose oxidase generating H_2O_2 at a rate of 4±0.3 nmol/min were added. After 30 min of incubation at 37°C the reactions were stopped by addition of 5% trichloroacetic acid (final concentration, wt/vol) for the estimation of the $^{14}CO_2$ formed. The amount of oxidized glucose was calculated as in Fig. 7. Values are means±1 SD of triplicate assays from one representative experiment.

Discussion

A series of clinical and experimental observations has raised the possibility that activated neutrophils may be the cause of vascular damage in pathologic conditions such as inflammation (37) and the shock lung syndrome (7, 8, 38, 39). For example, it has been shown that the release of lysosomal enzymes from activated neutrophils may cause detachment of cultured endothelial cells from the basal membrane (40, 41) and that the products of the oxidative metabolism of the neutrophils may affect endothelial cell viability. In the latter case, a role has been attributed to hydroxyl radical in the rat lung vascular damage induced by complement-activated neutrophils on the basis of the protection afforded by intraperitoneal injection of the hydroxyl radical scavenger dimethylsulfoxide (42). Instead, superoxide anion (O_2^-), another intermediate of the oxygen metabolism in neutrophils, does not seem to play a major role because superoxide dismutase, an enzyme that converts O_2^- to H_2O_2, has been reported to fail to protect cultured endothelial cells from lysis by activated neutrophils (1, 4) or by enzymatically produced O_2^- (5), while Hoover et al. (43) have reported an inhibition of O_2^- release by neutrophils exposed to cultured endothelial cells. Much attention has been placed on H_2O_2, the terminal product of the oxidative metabolism of neutrophils, as a potential toxic agent to the endothelial cells. In fact, catalase, an enzyme that reduces H_2O_2 to H_2O, prevents endothelial cell damage induced by activated neutrophils (1, 4, 5). On the other hand, both reagent grade H_2O_2 and enzymatically generated H_2O_2 have been described to affect adversely several cell functions of cultured en-

Table III. Hydrogen Peroxide Degradation and CO_2 Production from [1-^{14}C]- and [2-^{14}C]Glucose by NaN_3-inhibited Endothelial Cells

	nmol/10 min	*nmol/10 min*	*nmol/10 min*	*nmol/10 min*
1. Glucose oxidase-generated H_2O_2	7.5±0.1	18.5±0.1	37.5±0.4	87.5±1.0
2. H_2O_2 degraded	7.5±0.2	16.6±1.5	29.7±2.4	71.1±5.2
3. Oxidized glutathione	0.2±0.1	0.2±0.1	0.8±0.3	2.3±0.6
4. Difference (2–3)	7.3	16.4	28.9	69.4
5. CO_2 from [1-^{14}C]glucose (glucose equivalents)	2.2±0.8	3.4±0.4	6.5±0.5	11.0±0.2
6. CO_2 from [2-^{14}C]glucose (glucose equivalents)	0.1±0.01	0.2±0.1	0.7±0.1	1.6±0.01
7. Ratio total lactate/lactate from exogenous glucose	$\frac{81.3\pm11.0}{46.5\pm3.4} = 1.7$	$\frac{75.8\pm12.3}{37.0\pm4.3} = 2.0$	$\frac{66.5\pm5.3}{29.2\pm3.2} = 2.3$	$\frac{41.7\pm5.2}{16.1\pm3.0} = 2.6$
8. Total CO_2 formed ($5 \times 7 + 6 \times 7$)	3.9	7.2	16.6	32.8
9. Ratio H_2O_2 degraded/CO_2 formed (4/8)	1.9	2.3	1.7	2.1

10^7 endothelial cells were incubated for 10 min with varying amounts of glucose oxidase in 1 ml KRP containing NaN_3 (2 mM), 5.5 μmol unlabeled glucose, and 2 μCi of either [1-^{14}C]-, or [2-^{14}C]-, or [U-^{14}C]-labeled glucose. The glucose oxidase-generated H_2O_2 and the H_2O_2 degraded by endothelial cells were estimated fluorimetrically. The amount of oxidized glutathione is expressed as GSSG equivalents (1 nmol GSSG is equivalent to 2 nmol GSH). The total glutathione content (GSH + GSSG) of the samples was 8.5±1.0 nmol GSSG equivalents and remained constant during incubation with H_2O_2. The amount of CO_2 formed from [1-^{14}C]glucose and [2-^{14}C]glucose was calculated on the basis of an initial specific activity of extracellular glucose of 650 cpm/nmol. The ratio total lactate/lactate from exogenous glucose is used as a correction factor for the CO_2 formed from [1-^{14}C]- and [2-^{14}C]glucose, to allow for the contribution of the intracellular substrate pool to CO_2 formation (20). Total lactate production was determined enzymatically. Lactate from exogenous glucose was calculated from the radioactivity of extracts of samples incubated with [U-^{14}C]glucose, on the basis of an initial specific activity of 650 cpm/nmol. Values (nmol/10 min) are means±1 SD of triplicate samples from one experiment.

dothelial cells, the threshold of toxic H_2O_2 concentration varying according to the function explored (5). However, it is unclear whether the existence of a threshold reflects the degree of sensitivity of the index used to monitor cell functions or an active role of endothelial cells in H_2O_2 detoxification. In this article we present evidence that MEC are able to degrade H_2O_2 either produced by activated PMN or enzymatically, by glucose-glucose oxidase. With either H_2O_2-generating system, the amount of H_2O_2 degraded was about 4 nmol/min per 10^6 cells, corresponding to the H_2O_2 produced by about 3×10^6 PMA-stimulated bovine PMN during the initial linear phase of activation. We also show here that the two main pathways of H_2O_2 degradation in these cells are catalase and the glutathione redox cycle. Evidence for the role of catalase in H_2O_2 degradation by MEC is provided by the following findings: (*a*) high catalase activity in cell homogenates and (*b*) strong inhibition of the H_2O_2-degrading capacity of intact cells in the presence of the catalase inhibitor NaN_3. An important role of the glutathione cycle in protecting cultured endothelial cells from lysis by environmental H_2O_2 has been already reported by Harlan et al. (36). In that study, ^{51}Cr release from cultured endothelial cells obtained from human umbilical vein, bovine aorta, and pulmonary artery was not increased upon treatment with H_2O_2 generated by either the glucose-glucose oxidase system or PMA-stimulated human neutrophils. However, either pretreatment of the cultures with specific inhibitors of the glutathione cycle or depletion of endothelial cells glutathione caused cell lysis upon treatment with H_2O_2. Further evidence for a role of the glutathione redox cycle in protecting cultured endothelial cells isolated from the calf pulmonary artery against environmental H_2O_2 was recently revealed by Tsan et al. (6), who showed an inverse linear correlation between cell GSH levels and H_2O_2-mediated cytolysis. In the present study, we show that H_2O_2 degradation through the glutathione redox cycle is coupled in MEC with activation of the hexose monophosphate shunt. This is based on the following findings: (*a*) stimulation of $^{14}CO_2$ production from [1-^{14}C]- and [2-^{14}C]glucose by H_2O_2, (*b*) inhibition of this stimulation by the glutathione reductase inhibitor BCNU, (*c*) a 2:1 stoichiometry between the H_2O_2 degraded in the presence of NaN_3 through the glutathione redox cycle and the CO_2 generated by the shunt, and (*d*) maintenance of the reduced-glutathione levels in catalase-inhibited cells exposed to H_2O_2 concentrations below the H_2O_2-

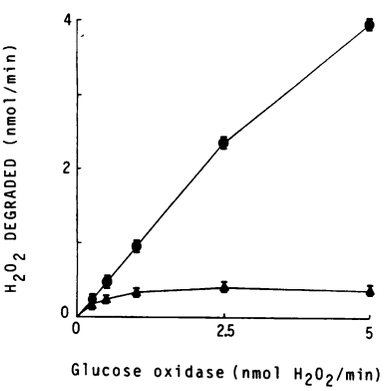

Figure 10. Relative role of catalase and of the glutathione redox cycle in degradation of glucose-glucose oxidase-generated H_2O_2 by endothelial cells. The conditions of incubation were the same as in Table II, except for the absence of NaN_3 and for the incubation time (30 min). The total H_2O_2 degraded (●) was estimated fluorimetrically; the H_2O_2 degraded by the glutathione redox cycle (▲) was calculated from the production of CO_2 from [1-^{14}C]- and [2-^{14}C]glucose, corrected for the dilution by unlabeled CO_2 originating from endogenous glucose, as described in Table II. Values (nmol/min per 10^6 cells) are means±SD of triplicate assays from a typical experiment.

degrading capacity of the glutathione redox cycle coupled with the HMPS and abrupt increase of the ratio of oxidized glutathione to reduced glutathione as soon as the H_2O_2 concentrations exceeded this capacity. The 2:1 stoichiometry between the H_2O_2 degraded and the CO_2 produced also indicates that the glutathione redox cycle is the only NaN_3-insensitive pathway of H_2O_2 degradation by MEC. In fact, a higher ratio would be expected if other NaN_3-insensitive H_2O_2-degrading mechanisms were available. In conclusion, only two H_2O_2-degrading pathways are present in these cells—one NaN_3-sensitive, catalase, and the other NaN_3-insensitive, the glutathione redox cycle. Hence it is possible to calculate the relative contribution of these two pathways in H_2O_2 degradation by MEC even in the absence of inhibitors: the amount of H_2O_2 degraded by the glutathione redox cycle is obtained by multiplying by 2 the CO_2 generated by the shunt whereas the H_2O_2 degraded by catalase is obtained from the difference between the total H_2O_2 degraded, as assayed directly, and the H_2O_2 degraded through the glutathione cycle. From this calculation it was concluded that the role of the glutathione cycle and of catalase varied depending on the environmental H_2O_2 concentration. For example, at an H_2O_2-generation rate of 0.25 nmol/min per 10^6 cells, the glutathione cycle accounted for about 75% of the H_2O_2 degraded. Instead, at higher H_2O_2 concentrations (above H_2O_2 generation rates of 0.5 nmol/min per 10^6 cells), the contribution of catalase became prominent, which is in agreement with the low affinity of catalase for H_2O_2 as compared with glutathione peroxidase (44).

It is important to note that MEC exposed to H_2O_2 concentrations in the range of their degradative capacity did not undergo relevant damage as judged by two different criteria, thus suggesting that H_2O_2 degradation is for endothelial cells an important means of self-protection against the oxidative stress. Accordingly, when the cells were exposed to H_2O_2 concentrations exceeding their degradative ability, cell damage became apparent. It seems therefore that the oxidative damage at sites of neutrophil activation is not an inevitable event, and that its occurrence and extent depends on the balance between the oxidative charge released by the neutrophils and the defensive potential of endothelial cells.

Although the experimental conditions used in this study are difficult to compare with those present in vivo at the inflammatory site, the present results suggest that the microvascular inflammatory lesions cannot be straightforwardly attributed to direct endothelial damage by H_2O_2. An interesting alternative explanation of the mode of action of H_2O_2 is suggested by the studies by Ager and Gordon (5) and Harlan and Callahan (45), who have shown that nonlytic doses of H_2O_2 induced prostaglandin release by endothelial cells, which could account per se for increased vascular permeability (46–48).

Acknowledgments

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