Mechanisms of Endothelial Cell Killing by H_2O_2 or Products of Activated Neutrophils

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Interactions between rat pulmonary artery endothelial cells and hydrogen peroxide or toxic oxygen products from phorbol ester-activated human neutrophils result in endothelial cell killing defined by ^{51}Cr release. It has been shown that this cytotoxic reaction can be blocked by the presence of catalase, iron chelators, or scavengers of the hydroxyl radical. Evidence shows that products from xanthine oxidase of endothelial cells are necessary for the toxic effects of hydrogen peroxide or phorbol ester-activated neutrophils. Addition of xanthine oxidase inhibitors protects against phorbol ester-mediated injury of endothelial cells. Preloading of endothelial cells with superoxide dismutase attenuates injury caused either by hydrogen peroxide or phorbol ester-activated neutrophils. Conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells occurs during contact of endothelial cells by activated neutrophils. This conversion is not related to oxygen products of neutrophils. Conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells is also induced by endothelial cell contact with C5a, N′-formyl-methionyl-leucyl-phenylalanine (fMLP), or tumor necrosis factor alpha (TNFα). Interaction of hydrogen peroxide with endothelial cells rapidly depletes adenosine triphosphate (ATP) and causes the extracellular appearance of xanthine and hypoxanthine. Agents that protect endothelial cells from the toxic effects of hydrogen peroxide do not prevent falls in cellular ATP caused by hydrogen peroxide, indicating that ATP levels do not necessarily correlate with cytotoxic events. A synergy between hydrogen peroxide and proteases in endothelial cell killing has been demonstrated. TNFα causes alterations in endothelial cells, the result of which is increased susceptibility to killing by PMA-activated neutrophils.

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This work was supported in part by USPHS grants GM-29507, HL-31963, and HL-07517.

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Injury of endothelial cells by activated neutrophils in vivo and in vitro is documented [1–6]. It appears that in vitro vascular endothelial cell killing by phorbol ester (PMA)-activated human neutrophils is related to the formation of toxic oxygen products coming from the respiratory burst of the neutrophils. Since catalase, deferoxamine, and hydroxyl radical (· OH) scavengers, such as dimethyl sulfoxide and dimethylthiourea, provide high degrees of protection to endothelial cells both in vitro and in vivo [4], it is suggested that · OH may be a key factor in the injury of endothelial cell killing by activated neutophils. The inability of myeloperoxidase (MPO) inhibitors to protect against in vitro endothelial cell killing by activated neutrophils suggests that the halide-dependent products of hydrogen peroxide (H_2O_2), such as hypochlorous acid (HOCl), are not involved in the injury process [4], although it is suggested that HOCl may play other roles, such as causing activation of the precursor forms of collagenase and gelatinase, the outcome of which would be breakdown of connective tissue matrix [7]. In this report, we review our own studies, which suggest that a complex series of events are involved in killing of rat pulmonary artery endothelial cells by H_2O_2 or by products of activated neutrophils.

PARTICIPATION OF XANTHINE OXIDASE IN ENDOTHELIAL CELL KILLING BY ACTIVATED NEUTROPHILS OR BY H_2O_2

Recent experiments suggest that xanthine oxidase (XO) present within rat pulmonary artery endothelial cells plays a role in the killing of these cells by PMA-activated human neutrophils [8]. These endothelial cells (but not neutrophils) contain both XO as well as its precursor, xanthine dehydrogenase (XD). When three different inhibitors (allopurinol, oxypurinol, and lodoxamide) of XO were present in the culture medium, there was a 29–64% reduction in endothelial cell killing reflected by their release of ^{51}Cr (**Table I**). The protective effects were dose dependent on the inhibitor present, showing the ability of these inhibitors to block the catalytic activity of endothelial cell XO [8]. If endothelial cells were pretreated with these inhibitors and then washed, the protective effects remained, whereas similar pretreatments of neutrophils did

TABLE I

Ability of Xanthine Oxidase Inhibitors to Protect Endothelial Cells from Cytotoxicity Due to Activated Neutrophils

Interactions*	Cytotoxicity (% Specific Release of ^{51}Cr)	Inhibition (%)
None	32.7 ± 1.7	—
Allopurinol	12.8 ± 2.1	60.8
Oxypurinol	11.9 ± 0.5	63.6
Lodoxamide	23.1 ± 1.1	29.3

Data from [8].
*Inhibitors were used at a concentration of 5 m*M*.

TABLE II

Ability of Superoxide Dismutase (SOD) to Protect Endothelial Cells from Injury by H_2O_2 or Activated Neutrophils

Injurious Agent	Preloading Concentrations of SOD*	SOD Activity in Endothelial Cells (Units/10^6 Cells)	Cytotoxicity (% Specific Release of ^{51}Cr)†
H_2O_2, 100 μM	None	12 ± 2	45 ± 7
H_2O_2, 100 μM	2.0 mg	49 ± 6	34 ± 2
H_2O_2, 100 μM	5.0 mg	168 ± 13	24 ± 6
H_2O_2, 100 μM	10 mg	187 ± 8	21 ± 7
Neutrophils, 0.8 × 10^6*	None		18 ± 2
Neutrophils, 0.8 × 10^6†	10 mg		8 ± 1

*SOD, bovine CuZn–superoxide dismutase, containing 11,028 ± 228 units/mg of protein. Human neutrophils were activated by addition of 100 ng/ml of phorbol myristate acetate.
†Data from [9].

not result in diminished endothelial cell killing (unpublished data). These data provide support for the hypothesis that use of XO in endothelial cells is somehow linked to the ability of activated neutrophils to kill these cells.

Direct evidence for the role of superoxide anion (O_2^-) in endothelial cell killing by H_2O_2 or PMA-activated neutrophils is shown in **Table II**. For these studies, neutrophils were incubated with varying concentrations of bovine CuZn–superoxide dismutase (SOD) for 18 hours, washed, and then exposed either to 100 μM H_2O_2 or to 8×15^5 neutrophils activated with PMA. The end points were ^{51}Cr release from endothelial cells. Data show a progressive increase in the amount of SOD activity present in cell extracts, proportional to the amount of SOD to which the endothelial cells have been exposed. It is also apparent that there was progressive inhibition in the release of ^{51}Cr subsequent to the addition of H_2O_2 as a function of the amount of SOD activity measurable in extracts of endothelial cells. Heat-inactivated SOD failed to provide any protection against H_2O_2-mediated killing [9]. As expected, the H_2O_2 preparation did not contain any measurable O_2^- (unpublished data), indicating that endothelial cells must be the source of the substrate for SOD. Also shown in Table II is the ability of endothelial cells, preloaded with SOD and exposed to PMA-activated neutrophils, to demonstrate 56% protection from killing because of increases in cellular SOD. In conclusion, endothelial cell killing following exposure either to H_2O_2 or PMA-activated neutrophils involves endothelial cell-derived O_2^-. It is believed that $O_2^- \cdot$ is produced from XO of the endothelial cell.

CONVERSION IN ENDOTHELIUM OF XANTHINE DEHYDROGENASE TO XANTHINE OXIDASE

Studies provide evidence that conversion in endothelial cells of XD to XO occurs by contact of endothelial cells with PMA-activated neutrophils [8]. This conversion process is irreversible (XO does not revert to XD following addition of reducing agents). **Figure 1** shows that agents (catalase, deferoxamine) that prevent endothelial cell killing by PMA-activated neutrophils [4] and SOD failed to interfere with the conversion of XD to XO. In this experiment, the percent of total activity (XD and XO) due to XO was approximately 40% when non-activated neutrophils (PMNs) were present, rising to nearly 70% when PMA stimulated the neutrophils. These data suggest that oxygen products of activated neutrophils do not account for the conversion of XD to XO in endothelial cells, supported by data in **Figure 2** in which PMA-treated neutrophils from a normal human donor and from a patient with chronic granulomatous disease of childhood (CGD) were used. Neutrophils from the patient donor are unable to exhibit a respiratory burst after addition of PMA and do not form H_2O_2 or O_2^-. Figure 2 shows no difference in the conversion of XD to XO when results from the two cell donors are compared, indicating that the XD "converting factor" of the activated neutrophil is not related to an oxygen product.

Table III shows that conversion of XD to XO occurs because of endothelial cell interaction with certain peptide mediators [10]. For the conversion process, the ED_{50} is shown for the compounds listed in Table III. Human C3a was inactive, but human recombinant C5a was highly active, with an ED_{50} of 4 n*M*. Enzymatic removal of the C-terminal arginine abolished the activity of C5a. The synthetic chemotactic peptide, N′-formyl–Met–Leu–Phe (fMLP), and recombinant human tumor necrosis factor α (TNFα) showed activity, but not recombinant human interleukin-1β (IL-1β) or bradykinin, which are reactive with endothelial cells [11–13]. Finally, phorbol ester (PMA) did not convert XD to XO. These findings demonstrate that certain chemotactic peptides can directly interact with endothelial cells and be functional. The mechanism is not clear, but the conversion process of XD to XO was rapid (complete within 5–10 minutes) and was irreversi-

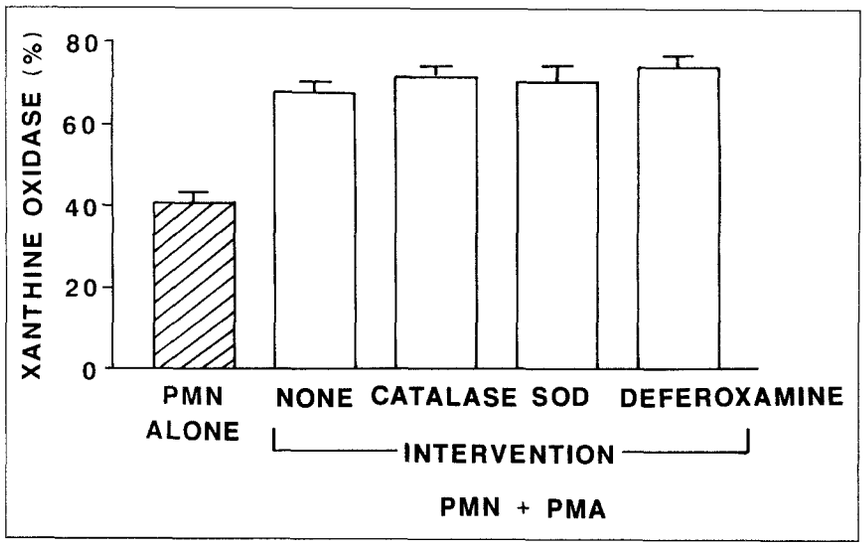

Figure 1. Percent xanthine oxidase (XO) activity present as combined catalytic activity of xanthine dehydrogenase (XD) and XO. Human neutrophils alone or in the presence of phorbol ester (PMA) were present in association with monolayers of rat pulmonary artery endothelial cells. After 4 hours, the cell monolayers were harvested and extracted. XD and XO were measured and the percent of total activity present as XO calculated. The data demonstrate that none of the interventions used [catalase, superoxide dismutase (SOD), and deferoxamine] blocked the conversion of XD to XO in endothelial cells exposed to phorbol ester-activated neutrophils. PMN: nonactivated neutrophils.

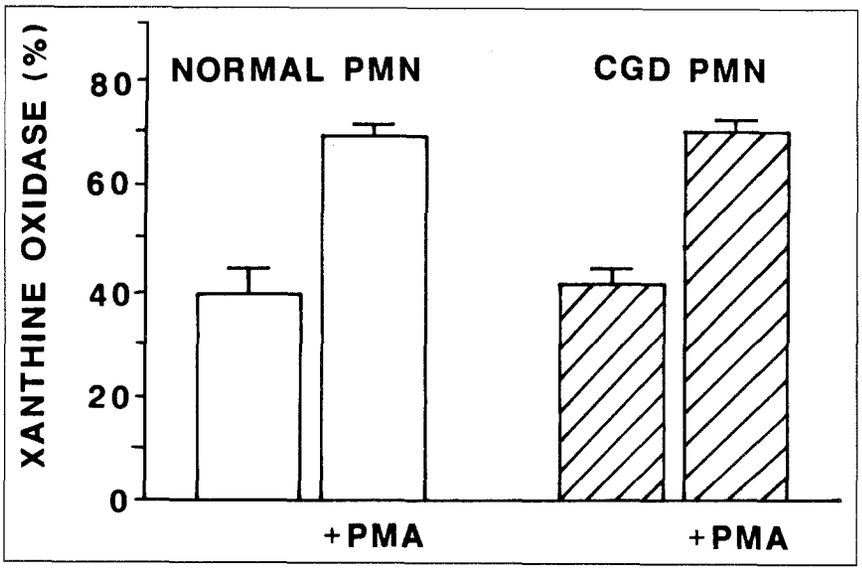

Figure 2. Comparison of the abilities of activated normal neutrophils and activated neutrophils from a patient with chronic granulomatosis disease of childhood (CGD) to convert XD to XO in rat pulmonary artery endothelial cells. As evident from the data, the amount of conversion occurring with activated normal neutrophils was the same as the amount of conversion occurring with activated CGD neutrophils. Hence, the converting factor cannot be linked to an oxygen product. PMN: nonactivated neutrophils.

ble [10]. These findings suggest that a change in the ratio of XD to XO in favor of XO places the endothelial cell at risk of injury, especially in the presence of H_2O_2 or activated neutrophils.

RELATIONSHIP OF ENDOTHELIAL CELL ATP AND INJURY PRODUCED BY H_2O_2

It is known that any type of cell injury results in loss of ATP levels within the affected cell [14–16]. In the endothelial cells, the fall in ATP after exposure has been postulated due to loss of ATP synthesis through inactivation of an enzyme in the Krebs cycle [14], associated with cell injury or death. **Table IV** addresses the following question: Does H_2O_2-induced loss of ATP correlate with cell death measured by ^{51}Cr release? For these experiments, endothelial cells were loaded with [^{14}C] adenosine, the bulk being converted to ATP, providing a radioactive pool for monitoring [17]. These studies measured the breakdown of the existing pool of [^{14}C] ATP. The experiments were carried out in the presence or absence of three agents (deoxycoformycin, allopurinol, and deferoxamine) providing 46–95% protection against H_2O_2-induced cytotoxicity (Table IV). Exposure of endothelial cells to H_2O_2 reduced [^{14}C] ATP by 72%. This reduction in ATP occurred within the first 20–30 minutes after addition of H_2O_2, and ATP levels remained at the reduced level for several hours. In all cases when cells were exposed to H_2O_2, there were significant amounts of xanthine (X) and hypoxanthine (HX) in the extracellular fluid (Table IV). These levels reduced by nearly one-half (to 9%) in the presence of deoxycoformycin, an adenosine deaminase inhibitor. Deoxycoformycin increased levels of cellular adenosine monophosphate (AMP). The remarkable aspect of these data was the fact that there was little or no sparing effect in levels of ATP by agents

TABLE III

Structure–Function Specificity of Peptides in Conversion of Xanthine Dehydrogenase (XD) to Xanthine Oxidase (XO) in Endothelial Cells

Material Used	ED_{50} for Conversion of XD to XO
C3a	>5 μM
C5a	4 nM
C5a desarg	>1 μM
fMLP	46 nM
TNFα	12 nM
IL-1β	>10 units
Bradykinin	>10 μM
PMA	>10 μM

Data from [10].

providing high degrees of protection against the cytotoxic effects of H_2O_2. The protective effects of deferoxamine, allopurinol, and deoxycoformycin are not related to maintaining high cellular levels of ATP in H_2O_2.

SYNERGY BETWEEN PROTEASES AND H_2O_2 IN ENDOTHELIAL CELL CYTOTOXICITY

Although antiproteases, such as soybean trypsin inhibitor, do not protect endothelial cells against in vitro cytotoxic effects of PMA-stimulated neutrophils [4], it is likely that proteases and toxic oxygen products from neutrophils may jointly contribute to cytotoxic effects of activated neutrophils on vascular endothelial cells. There could also be synergistic effects of H_2O_2 and protease on matrix substances, such as vascular basement membrane. **Table V** shows that compared with H_2O_2-induced cytotoxicity of endothelial cells, there is a synergy between protease actions and H_2O_2 effects. The experiments involved an end point of 18 hours during which the cytotoxic effects of H_2O_2 were observed [18]. Cells were exposed to 0.1 mM H_2O_2 for 18 hours, protease for 18 hours, or to both (protease for the first 2 hours, and then addition of H_2O_2 and termination of the experiment at 18 hours). Table V shows that, regardless of which of the three proteases was used, there was limited cytotoxicity with cell exposure either to H_2O_2 or to protease; the dual exposure of endothelial cells to protease and H_2O_2 resulted in cytotoxicity, which increased in a synergistic manner. The mechanisms of these synergistic cytotoxic effects are not known, but data show that alteration of endothelial cells by exposure to protease exposes them to intensified injury by the addition of H_2O_2. Whether or not exposure to H_2O_2 increases the permeability of endothelial cells and permits entry of the proteases with resulting proteolytic conversion of XD to XO is a possible explanation for the effects.

ENHANCEMENT OF NEUTROPHIL-MEDIATED CYTOTOXICITY OF ENDOTHELIAL CELLS BY TUMOR NECROSIS FACTOR ALPHA

It is known that exposure of endothelial cells to cytokines, such as TNFα or IL-1β, results in a time-dependent (requiring several hours) and a cycloheximide-sensitive change related to increased adherence of human neutrophils to the cytokine-altered endothelial cells [13]. We analyzed the extent to which TNFα may induce other functional changes in endothelial cells, such as increased sensitivity to the cytotoxic effects of activated neutrophils. Endothelial cells were exposed to human recombinant TNFα for 5 or 18 hours, washed, and then neutrophils added followed by addition of PMA. Cytotoxicity was measured after 4 hours [19]. **Table VI** shows that in endothelial cells not treated with TNFα, PMA-activated neutrophils caused 17% cytotoxicity. With prior exposure of endothelial cells to TNFα for 18 hours followed by washing and addition of neutrophils, but in the absence of PMA, cytotoxicity was negligible (2%). If endothelial cells were exposed to TNFα for 5 to 18 hours, subsequent addition of PMA-activated neutrophils resulted in cytotoxicity ranging from 31–41%. Use of heat-inactivated TNFα (100°C for 20

TABLE IV

Changes in Endothelial Cell ATP in Presence or Absence of Protective Agents

			Metabolite (% of Total Radioactivity)					
			Cellular			Extracellular		
Condition*	Intervention†	Protection (%)‡	ATP	AMP	X/HX	ATP	AMP	X/HX
Buffer	None	—	60 ± 13	3 ± 3	<1	<1	<1	1 ± 1
H_2O_2	None	0	17 ± 7	4 ± 1	3 ± 1	<1	<1	15 ± 7
H_2O_2	Deferoxamine	95 ± 3	23 ± 11	3 ± 1	2 ± 1	<1	<1	15 ± 5
H_2O_2	Allopurinol	65 ± 10	24 ± 2	3 ± 1	5 ± 3	<1	<1	17 ± 5
H_2O_2	Deoxycoformycin	46 ± 6	19 ± 3	20 ± 9	3 ± 3	<1	<1	9 ± 2

*When present, 0.5 mM H_2O_2 was used. ATP and related metabolites were measured 30 minutes after addition of H_2O_2.
†Concentrations of protective drugs employed were 1 mM in each case.
‡Defined as percentage reduction in specific release of ^{51}Cr from endothelial cells 4 hours after addition of H_2O_2.
Adapted from [17].

TABLE V

Synergy Between Proteases and H_2O_2 in Endothelial Cell Killing

Experiment	Material Added to Endothelial Cells*	Cytotoxicity (% Specific Release of ^{51}Cr)†
A	H_2O_2 (0.1 μM)	33 ± 1
	Pancreatic elastase (2.3 units)	13 ± 1
	Both	75 ± 2
B	H_2O_2	44 ± 4
	Cathepsin (2.5 μg)	5 ± 2
	Both	76 ± 1
C	H_2O_2	23 ± 2
	Chymotrypsin (3.0 units)	5 ± 1
	Both	48 ± 3

*Cells were exposed to H_2O_2 and/or to protease for 18 hours, following which ^{51}Cr release was measured. When both reagents were present, H_2O_2 was added, followed by addition of protease 2 hours later.
†Data from [18].

TABLE VI

Ability of TNFα to Enhance Endothelial Cell Killing by Activated Human PMNs*

Pretreatment of Endothelial Cells	Neutrophil Treatment	Cytotoxicity (% Release of ^{51}Cr)
None	None	<1
None	PMA (100 ng/ml)	17 ± 5
TNFα, 18 hours (50 ng/ml)	None	2 ± 1
TNFα, 5 hours	PMA	31 ± 5
TNFα, 18 hours	PMA	41 ± 6
TNFα, 18 hours heat inactivated	PMA	19 ± 5
None	TNFα (30 minutes pretreated, followed by PMA)	12 ± 7

*Results from [19].

minutes) abolished the ability of the cytokine to modify endothelial cells, rendering them more sensitive to the cytotoxic effects of PMA-activated neutrophils. Addition of TNFα to the neutrophils followed by their washing and addition of PMA did not increase endothelial cell killing [19]. ^{51}Cr release from endothelial cells was reduced from 17% to 12%, apparently due to a "deactivation" of the neutrophils.

These data show that cytokine-induced changes of endothelial cells render these cells more susceptible to the injurious effects of PMA-activated neutrophils. It has also been shown that, under these conditions, the bulk of the cytotoxic effects of activated neutrophils can be blocked either by catalase or deferoxamine [19]. The mechanism of increased cytotoxicity, due to activated neutrophils, apparently operates through the same pathway normally used when PMA-activated neutrophils are in contact with endothelial cells not pretreated with TNFα.

Data in this review show that, in the interaction between activated neutrophils and endothelial cells, endothelial cells are not passive partners in the events leading to cytotoxicity. Endothelial cell generation of $O_2^- \cdot$ is apparently a key event resulting in cytotoxicity following exposure to PMA-activated neutrophils (or after exposure to H_2O_2). In rat pulmonary endothelial cells, O_2^- is apparently derived from XO, and its activity is increased by endothelial cell contact with PMA-activated neutrophils or by contact with chemotactic mediators such as C5a, TNFα, or fMLP. The main role of H_2O_2 may be to break down ATP, such that the substrates for XO (xanthine and hypoxanthine) become available. Apparently, $\cdot OH$ causes maximal damage resulting in cytotoxic changes in endothelial cells, although debate surrounds whether $\cdot OH$ is a product of the Fenton reaction or derived from a more recently described interaction between $O_2^- \cdot$ and nitric oxide [20]. The findings of the synergy between proteases and H_2O_2 in endothelial cell killing suggest other mechanism(s), showing endothelial cell killing by neutrophils and their products. These observations provide new insights into the multiple steps associated with cytotoxic events in endothelial cells because of their contact with activated neutrophils. The data suggest new therapeutic approaches for the protection of vascular endothelial cells in acute inflammatory reactions.

ACKNOWLEDGMENT

I wish to acknowledge the invaluable contribution of Kimberly D. Lowe in the preparation of this manuscript and to Dr. James Varani and Dr. P. Phan for their valuable collaborative contribution and availability of information to enable its review.

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