Mechanism of Endothelial Cell Shape Change in Oxidant Injury

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Changes in endothelial cell morphology induced by neutrophil-generated hydrogen peroxide (H₂O₂) may account for the capillary leak of the adult respiratory distress syndrome (ARDS). The relationship of H₂O₂ effects on the concentration of intracellular Ca²⁺([Ca²⁺]) and ATP to changes in microfilaments and microtubules, important determinants of cell shape, was examined. Bovine pulmonary artery endothelial cells were injured over a 2-hr time course with a range of H₂O₂ doses (0–20 mM). The higher concentrations of H₂O₂ consistently produced contraction and rounding of >60-76% of cells by 1–2 hr. The range of 1–20 mM H₂O₂ produced rapid, significant reductions in endothelial ATP levels over the time course of injury. Although there were significant increases in mean endothelial [Ca²⁺] in response to 5, 10, and 20 mM H₂O₂, 1 mM H₂O₂ did not affect the [Ca²⁺]. Fluorescence microscopy revealed that microfilament disruption occurred as ATP levels fell and preceded depolymerization of microtubules which developed after [Ca²⁺] approached 1 × 10⁻⁶ M. H₂O₂ at 1 mM injury caused microfilament disruption but did not depolymerize microtubules. Microfilament disruption occurred without oxidant exposure, when ATP levels were reduced by glucose depletion and mitochondrial inhibition with oligomycin (650 nM). If a Ca²⁺ ionophore, ionomycin (5 μM), was then added, [Ca²⁺] rose to >1 × 10⁻⁶ M, microtubules fragmented and depolymerized, and cell contraction and rounding very similar to that induced by H₂O₂ occurred. These results suggest that endothelial cell dysfunction and capillary leak in ARDS may be due to H₂O₂-mediated changes in cellular ATP and [Ca²⁺].

INTRODUCTION

Endothelial cells form a major part of the normal capillary permeability barrier. Changes in endothelial cell shape which occur during inflammation (e.g., ARDS) have been associated with increased capillary permeability [1–3]. Work with a variety of cell types including endothelial cells has demonstrated their ability to form tight junctions and act as permeability barriers when grown as monolayers on micropore filters [4–7]. The model permeability barriers lost their functional integrity when exposed to agents causing cell injury. Activated polymorphonuclear leukocytes (PMN) [9], superoxide anion (O₂⁻) [7], H₂O₂ [8], the Ca²⁺ ionophore A23187 [7], and cytochalasins [5, 6] were capable of disrupting the in vitro permeability barriers. Cytochalasins and activated PMN, which produce a number of different oxidants including O₂⁻, hydroxyl radical (OH·), hypohalous acids, and H₂O₂ [6, 9] have also been shown to be capable of causing capillary leak in isolated perfused lungs. Constant infusion of micromolar quantities of H₂O₂ in an isolated-perfused lung model also produced significant capillary leak over a 90-min time course [10]. Injury in the model systems was associated with cytoskeletal changes and gap formation between cells which correlated with loss of transmembrane electrical resistance and increased permeability of the monolayers to albumin [4–7].

Two major cytoskeletal structures involved in maintaining cell shape are microfilaments and microtubules.

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Developed as the injury progressed [19]. The microfilamentation of ATP levels in target cells by inhibition of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, and inhibition of mitochondrial ADP phosphorylation [15, 16]. Intracellular Ca²⁺ levels in the P388D₁ cell line also rose in response to H₂O₂ injury [17]. DNA damage with strand breakage and activation of the nuclear enzyme poly(ADP) ribose polymerase occurred rapidly following H₂O₂ exposure [18]. Gross changes in surface morphology (membrane blebbing) and disruption of the cytoskeleton with formation of microfilament bundles developed as the injury progressed [19]. The microfilament disruption into bundles was found to be an ATP-dependent phenomenon which could be reproduced by metabolic inhibition without oxidant exposure [20, 21].

The approximate threshold level of ATP in endothelial cells for microfilament disruption and bundle formation was 15–20% of normal levels [20]. The ATP-dependent changes in microfilament organization were independent of any net change in polymerized actin within the cell [21]. The microfilament bundle formation was reversible if ATP synthesis could be restored [20].

Holman et al. [22] recently demonstrated with an in vitro permeability model that oxidant-induced increases in permeability were associated with a fall in endothelial cell ATP levels.

The relationship between oxidant-mediated effects on ATP levels and intracellular Ca²⁺ regulation and the cytoskeleton is unclear. We examined the effect of a range of H₂O₂ doses given as a single bolus on ATP levels and the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in bovine pulmonary artery endothelial cells. These relationships were then correlated to concurrent changes in the organization of microfilaments and microtubules and associated morphology in injured cells. The effects of altering endothelial ATP and [Ca²⁺]ᵢ, independent of oxidant injury on cytoskeletal organization and cellular morphology were also examined.
METHODS

Cells and Culture

Bovine pulmonary artery endothelial cells (No. AG 2791 A) from the National Institute of Aging, Aging Cell Culture Repository (Camden, N.J.) were grown to confluence in 150-cm² flasks (Corning). They were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Whittaker, M.A. Bioproducts), 2 mM glutamine (GIBCO), 10 mM Hapes (Whittaker, M.A. Bioproducts), 100 μM penicillin, and 100 μg/ml streptomycin (GIBCO). Cells were passaged after a brief exposure (10–15 min) to trypsin (0.05%) and EDTA (0.02%; Sigma). The cells were suspended at a concentration of 2 × 10⁶/ml in modified Gey’s buffer (MGB) containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH₂PO₄, 1.1 mM Na₂HPO₄, 0.3 mM MgSO₄, 1 mM MgCl₂, 5.5 mM glucose, 10 mM Hepes, and 1.5 mM CaCl₂. In some experiments, glucose was absent from the buffer.

Experiments with suspended cells were carried out at 37°C in a shaking water bath.

Glutathione (GSH) Measurements

Measurement of total GSH was performed using the method of Brehe and Burch [23]. Briefly, 5 × 10⁶ cells were centrifuged for 30 sec in a microcentrifuge, the supernatant was removed, and the pellets were deproteinized with 200 μl 2.5% sulfosalicylic acid in 0.2% Triton X-100 for 3 min. Following another centrifugation, determinations of total acid soluble GSH were made in plastic cuvettes using 25-μl samples after addition of 500 μl 0.3 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 500 μl 0.4 mM NADPH containing 0.12 μl glutathione reductase, and 500 μl phosphate/imidazole buffer, pH 7.2. The difference in optical density at 412 nm was read on a Gilford Model 250 spectrophotometer.

ATP Determinations

Endothelial cell ATP levels were measured by the luciferase–luciferin method of Stanley and Williams [24] as previously adapted [15, 21]. The luciferase–luciferin (Sigma No. LO633) was reconstituted at 40 mg/ml in sterile water and diluted 1:50 in a buffer containing 1% bovine serum albumin, 20 mM glycine, and 2 mM EDTA, pH 8.0. Measurements were performed in a LKB model 1251 automated luminometer. ATP measurements were normalized so they could be expressed as the mean ATP level per cell.

Measurement of [Ca²⁺] with Quin 2

Endothelial cells in suspension were loaded with the acetoxy–methyl ester of Quin 2 (Quin 2 AM; Amersham, Arlington Heights, IL) after the method of Tsien et al. [25] as previously modified [17]. Aliquots of 5 mM Quin 2 AM (reconstituted in DMSO) were added to gently stirred cells (10 μl/10⁶ cells/ml) at 1 μl/sec and then incubated on a rotational device at 37°C for 25–30 min until maximal uptake was achieved. After a wash step using MGB and a brief (5 min) centrifugation at 1500 rpm, the cells were resuspended in MGB at 2 × 10⁶ cells/ml.

Quin 2 fluorescence was read on a spectrofluorometer (Perkin Elmer LS5; modified for work with thermostatted, stirred cell suspensions) with an excitation wave length of 340 nm and an emission wave length of 520 nm. Fluorescence intensity (F) was measured directly on 1.5-ml stirred samples at 37°C. Maximum fluorescence (Fₘₐₓ) was determined after detergent lysis (10 μl/ml, 1% Triton X-100). Minimal fluorescence (Fₘᵢₙ) was measured after the addition of 50 μl of 0.7 M EGTA to chelate all Ca²⁺ present. [Ca²⁺] was determined using the following equation: [Ca²⁺] = 1.15 × 10⁻⁹ · (F - Fₘᵢₙ)/(Fₘₐₓ - Fₘᵢₙ). Quin 2 measurements of [Ca²⁺] become nonlinear at concentrations >1 μM or saturations of the probe ≥90% (Quin 2 saturation = (F - Fₘᵢₙ)/(Fₘₐₓ - Fₘᵢₙ) × 100%). Reasonably accurate measurement of [Ca²⁺] can thus be made up to 1 μM. Elevations of [Ca²⁺] above this level measured up to 10 μM. At these concentrations, above this level measured up to 10 μM.

Fluorescence Microscopy of Adherent Endothelial Cells

Microfilaments. Endothelial cells were grown in 6-well plates (Nunc) overnight at an approximate density of 1–2 × 10⁶ cells/cm². Injury was carried out in MGB after removal of the culture media present. Staining of adherent endothelial cells with fluorescent phalloidin [19, 26] was performed as follows: a cocktail with final concentrations of 50 μg/ml lysophosphatidyl choline, 1.85% phosphate-buffered formalin, and 165 nM rhodamine phalloidin in MGB was added to the cells washed free of media and then incubated for 10 min at 37°C or 20 min at room temperature. After another wash step, a glass coverslip was sealed to the monolayer with a small drop of 90% glycerol.

Microtubules [27]. Adherent endothelial cells prepared as for the morphology experiments described above were fixed with 2% paraformaldehyde at 37°C and then left on ice for 1 hr. After washing, the cells were permeabilized with 0.1% Triton X-100 for 3 min. Following a second wash step with MGB containing 1 mg/ml bovine serum albumin (Sigma No. 6003), the cells were exposed to 100 μl of a 1:30 dilution of the first antibody (rabbit antitubulin; ICN, Immunobiologicals, Lisle, IL) for 20 min at room temperature. A third wash step was performed and the fluorescent antibody (rhodamine-conjugated goat anti-rabbit IgG; Boehringer–Mannheim, Indianapolis, IN) was added for another 20-min incubation. A coverslip was then sealed to the monolayer with a small drop of 90% glycerol.

In some experiments in which double staining of the same cells for both microfilaments and microtubules was done, 165 nM NBD-phallacidin was added to the cells after fixation and permeabilization for 20 min at room temperature prior to double antibody staining. Stained
samples were then viewed with a Nikon optiphot fluorescence microscope. Fluorescence micrographs at either 400 or 1000X magnification were taken using plus X pan or Tmax film (Kodak).

RESULTS

Endothelial GSH as a Measure of Oxidant Stress

To demonstrate that an increasing oxidant load was being experienced by the endothelial cells over the dose range studied, total GSH 60 min after addition of H₂O₂ was measured (Fig. 1). A dose-dependent loss of total GSH was seen following exposure to H₂O₂ (P = 0.0002, one-way analysis of variance). Significant reduction of total GSH as compared with control (P < 0.05, t test) was seen with concentrations of H₂O₂ ≥ 5 mM.

Endothelial Cell ATP

ATP levels in bovine pulmonary artery endothelial cells fell rapidly in response to the range (1–20 mM) of H₂O₂ doses. Significant dose- and time-dependent reductions of ATP (P < 0.0001, two-way analysis of variance) were seen with all concentrations of H₂O₂ tested (Fig. 2). There was a tendency toward partial recovery of ATP levels in cells exposed to 1 mM H₂O₂ at later time points.

Endothelial Cell Ca²⁺ Homeostasis following Injury

Uninjured endothelial cells loaded with Quin 2 had a resting level of [Ca²⁺]i of approx 200 nM. There were significant increases in mean [Ca²⁺]i following exposure to 5, 10, and 20 mM H₂O₂ after 60 min (P < 0.05, Welch’s solution to the Behrens–Fisher problem of unequal variances) [28] (Fig. 3). H₂O₂ at 20 mM caused a rapid elevation in mean [Ca²⁺]i of the entire population of cells to levels (>1 μM) after 45–60 min beyond accurate measurement with Quin 2. Although 1 mM H₂O₂ produced a significant reduction in cellular ATP levels, it did not alter [Ca²⁺]i, from the control range (Fig. 3). H₂O₂ at 5 and 10 mM produced significant elevations of mean [Ca²⁺]i later than 20 mM H₂O₂ which produced significant elevation of [Ca²⁺]i, as early as 30 min after exposure (P < 0.003; Fig. 3).

When cellular ATP levels were reduced by glucose depletion and 650 nM oligomycin to a range similar to that induced by H₂O₂, [Ca²⁺]i, measured by Quin 2 after 1 hr was not significantly altered (0.155 ± 0.062 × 10⁻⁶ M, N = 4 versus 0.200 ± 0.055 × 10⁻⁶ M, N = 14 for the control, P > 0.05). Addition of 5 μM ionomycin to the metabolically inhibited endothelial cells loaded with Quin 2 consistently produced rapid, sustained elevation of [Ca²⁺]i to >1 μM by 1–2 min. Ionomycin alone without prior ATP depletion produced only transient elevations of [Ca²⁺]i, lasting for approx 5 min or less.

Cytoskeletal and Morphologic Changes Associated With Injury

Exposure to H₂O₂ led to dramatic changes in the organization of the endothelial cell cytoskeleton and cellular morphology. After exposure to H₂O₂ doses producing a significant decline in ATP levels and significant elevation of mean [Ca²⁺]i, there was a rapid disruption of microfilaments into shorter brightly staining aggregates followed by breakup and depolymerization of microtubules. Cell rounding or contraction occurred following disruption of these cytoskeletal structures (Fig. 4). H₂O₂ at 1 mM produced some microfilament disruption but no effect on microtubules over the time course of injury (Fig. 4). As a consequence of its lack of effect on [Ca²⁺]i and microtubule organization, 1 mM H₂O₂ caused only subtle changes in endothelial morphology.

When ATP levels were reduced with glucose depletion and exposure to 650 nM oligomycin as shown previously [20], no effect on microtubule organization was seen but microfilament disruption occurred which was very similar if not identical to that seen with H₂O₂ injury (Fig. 5). This was consistent with the observation of no change in [Ca²⁺]i, under similar conditions in Quin 2-loaded cells.

When 5 μM ionomycin, a Ca²⁺ ionophore, was added to the metabolically inhibited cells, rapid disruption and depolymerization of microtubules occurred followed by cell contraction and rounding (Fig. 5). When metabolic inhibition was combined with colchicine (5 μg/ml), which also depolymerizes microtubules, a very similar sequence of events occurred (data not shown). The addition of ionomycin to cells already treated by metabolic inhibition and colchicine did not further enhance the apparent rate of cell rounding/contraction (data not shown).

DISCUSSION

H₂O₂ exposure produced dose-dependent reductions of GSH and ATP and elevation of mean [Ca²⁺]i in bovine...
pulmonary artery endothelial cells. It is interesting to note that doses of H₂O₂ which produced significant reductions in total GSH also caused significant elevation of [Ca²⁺].

The close correlation between H₂O₂-induced reduction of ATP levels and microfilament disruption (particularly with 1 mM H₂O₂ where no change in [Ca²⁺], occurred) is also strongly supported by earlier work with metabolically inhibited endothelial cells in which a reversible ATP-dependent disruption of microfilaments was demonstrated [20]. The observation that [Ca²⁺]; is remarkably stable for at least an hour in metabolically inhibited cells suggests that something more than reduction of ATP levels is necessary to cause the rapid loss of [Ca²⁺], homeostasis seen following H₂O₂ exposure at the higher doses. Earlier work [17] with the P388D₁ cell line demonstrated an initial loss of Ca²⁺ from injured cells in the first few minutes following oxidant exposure, suggesting that H₂O₂ may be capable of inducing release of Ca²⁺ from an oxidant-sensitive intracellular store.

[Ca²⁺]; elevation induced by the higher doses of H₂O₂ followed the decline in ATP levels and was correlated closely with the breakup and ultimate depolymerization of microtubules. Although some subtle morphologic changes were seen with microfilament disruption alone, cell contraction and rounding with substantial loss of apparent surface area did not occur unless microtubule depolymerization accompanied the loss of normal microfilament architecture. It is interesting that conditions (e.g., metabolic inhibition) causing microfilament disruption in the P388D₁ cell type which were sufficient to induce cell rounding and contraction [21] did not alter cell shape to the same degree in endothelial cells. Microtubules probably play a more dominant role in maintaining endothelial cell shape than in the P388D₁ cell line.

To completely disrupt [Ca²⁺]; in the entire endothelial cell population, a relatively large dose (20 mM) of H₂O₂ was required. Since activated PMN release H₂O₂ over very short distances after adhering to endothelial cells, it is possible that the local concentration of H₂O₂ at the PMN–endothelial interface may be as high as the millimolar range. It is clear from the effect of 5 and 10 mM H₂O₂ on mean [Ca²⁺]; that there is probably a spectrum of [Ca²⁺]; from the control range to >1 μM in individual cells of the injured population. This implies that individual cells may be responding to H₂O₂ with different susceptibilities or that equivalent H₂O₂ doses may be delivered inefficiently to each cell in the population when given as a single bolus to the buffer containing the cells.

Sustained elevation of [Ca²⁺]; has been associated with irreversible cell injury [29]. It is interesting to note that a lower dose (1 mM) H₂O₂ injury could produce a significant reduction in endothelial cell ATP levels without affecting [Ca²⁺];, implying that it may represent an ultimately reversible injury. The reduction in ATP levels was still sufficient to induce some microtubule disruption. Since microfilaments may play a crucial role in cell–cell and cell–basement membrane adherence [11, 12], it is conceivable that even a reversible oxidant injury may be capable of transiently disrupting the barrier function of the capillary endothelium. This concept may be supported by the observation of Holman et al. [22] that reversible disruption of a model permeability barrier could be induced by oxidant exposure. More massive injury as reflected by the data from the higher H₂O₂ doses would presumably be associated with a greater degree of barrier disruption for a longer period of time.

Two important areas of focus for therapy may relate to (1) enhancement of intracellular antioxidant defenses such as augmentation of cellular GSH levels [30] and (2) maintenance or restoration of cellular ATP levels [31]. Increased antioxidant defenses such as higher GSH levels in the target cell might convert a potentially irreversible injury with elevated [Ca²⁺]; to one with only a transient reduction in ATP levels and a moderate, temporary impairment of function. Maintenance or restoration of ATP levels in injured cells may limit further loss of function by restoring normal microfilament architecture and by maintaining normal Ca²⁺ pump activity at the cell membrane which would help prevent Ca²⁺-induced depolymerization of microtubules. Figure 6 outlines in schematic form some of the metabolic consequences of H₂O₂ injury and their relationship to alterations in the endothelial cell cytoskeleton and their overall effect on cell shape.

In summary, H₂O₂ injury induced a rapid fall in en-
endothelial cell ATP levels which correlated closely with disruption of microfilaments into shortened aggregates. \([\text{Ca}^{2+}]_i\) elevation followed the \(H_2O_2\)-induced decline in ATP levels at higher doses of oxidant and was associated with microtubule depolymerization. The loss of both cytoskeletal structures was then followed by dramatic cell contraction and rounding. These observations may suggest important targets for future therapy.

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