Oxidants, ATP Depletion, and Endothelial Permeability to Macromolecules

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Oxidants can reversibly increase the permeability of endothelium to ions and macromolecules. Oxidants also deplete ATP in cultured endothelial cells. We asked if oxidant-mediated ATP depletion, alone, accounted for the effects of oxidants on endothelial permeability to macromolecules. When porcine pulmonary artery endothelial cells were exposed to 2.5 mmol/L H$_2$O$_2$, ATP was depleted to 31.7% ± 1.8% of control within 15 minutes and was reduced to 23.1% ± 2.0% of control after 30 minutes. To determine if this magnitude of ATP depletion could account for the oxidant-induced increase in endothelial permeability to macromolecules, we measured ATP in endothelial cells exposed to metabolic inhibitors of ATP production. We then measured the effects of these metabolic inhibitors on endothelial monolayer permeability to macromolecules. ATP levels were reduced to 44% ± 4% of control by 12 mmol/L deoxyglucose (DOG) in the absence of glucose and to 2% ± 1.3% of control by DOG with 25 nmol/L antimycin A in the absence of glucose. Reduction of endothelial cell ATP to these levels with the metabolic inhibitors did not alter the flux of albumin or dextran across the endothelial monolayers. Thus ATP depletion, by itself, does not explain oxidant-induced changes in endothelial permeability to macromolecules.

INCREASED PERMEABILITY of the microvasculature is an important characteristic of acute inflammatory edema. Granulocytes are believed to play an important role in this process, and reactive oxygen molecules (oxidants) released from granulocytes have been shown to increase endothelial permeability in vitro and in vivo.

ATP depletion occurs in endothelial and other cells exposed to oxidants. The precise mechanisms responsible for ATP depletion have not been determined, but several pathways are probably involved. The potential significance of ATP depletion in oxidant injured endothelial cells to the pathogenesis of acute inflammatory edema is also not known. Holmsen found that H$_2$O$_2$ depleted ATP in platelets, but that ATP depletion to the level caused by H$_2$O$_2$ did not disrupt platelet function if the adenylate energy charge were not altered. Wysolmerski reported that ATP depletion with deoxyglucose and antimycin A caused subtle changes in the staining pattern of the peripheral band of actin in cultured endothelial cells. The same ATP depletion prevented the cells from contracting after exposure to histamine, cytochalasin B or etchthlychorynol. Bolin et al blocked glycolytic ATP production in perfused rabbit lungs and found that inhibition of glycolysis did not alter the hydraulic conductivity of the lung vasculature.

From such scant information it is not possible to predict what the effects of oxidant-mediated ATP depletion might be on endothelial permeability to macromolecules. In these experiments we measured the extent of oxidant-induced ATP depletion in porcine pulmonary artery endothelial cells, cultured the same as the cells we previously used to observe reversible oxidant-induced increases in endothelial permeability to albumin. We then caused similar levels of ATP depletion in the same cells with deoxyglucose and antimycin A and asked if these levels of ATP depletion altered the flux of albumin or dextran across monolayers of the same cells.

MATERIALS AND METHODS

Materials. Materials were obtained form the following sources: H$_2$O$_2$, trichloroacetic acid (TCA), and phosphoric acid from Fischer Scientific, Pittsburgh, PA; antimycin A (AA), dextran (average molecular weight 67,900), glucose, deoxyglucose (DOG), bovine serum albumin (BSA, fatty acid free), luciferin-luciferase, and fetal bovine serum (FBS) from Sigma Chemical, St Louis, MO; catalase was also from Sigma and was pretreated with polymyxin B agarose prior to use; $^{14}$C albumin from New England Nuclear, Boston, MA; $^{3}$H dextran from Amersham, Arlington Heights, IL; glucose free Earle's balanced salt solution (EBSS-GF) was made in the laboratory using the standard ion constituents for Earle's balanced salts; Medium 199 (M199) and trypsin-EDTA were from the Cancer Center, University of Iowa.

Cell culture. Porcine pulmonary arteries were obtained immediately after slaughter at a local abattoir. Resected ends of the arteries were clamped, and the artery segment was quickly dipped in 70% ethanol, then immediately rinsed in M199 with penicillin (100 U/mL) and streptomycin (100 μg/mL) (1 × P + S). Arteries were then unclamped and placed in sterile M199 with 1 × P + S. In the laboratory the arteries were opened longitudinally and the lumen gently scraped with a scalpel blade or a cotton tipped applicator. Cells were released into 35-mm-diameter tissue culture plates with Medium 199 supplemented with Basal Medium Eagle (BME) vitamins (GIBCO, Grand Island, NY) and amino acids, 1 × P + S, 10% FBS and then incubated at 37°C in 5% CO$_2$ and 95% air. Cells were subcultured 3:1 using trypsin-EDTA twice weekly, and cultures from passages 4 through 10 were used. Cells determined to be endothelial by morphology and by uptake of acetylated low density lipoprotein.

ATP measurements. Porcine pulmonary artery endothelial cell monolayers were exposed to H$_2$O$_2$ in M199 or to combinations of metabolic inhibitors with and without glucose in EBSS. Monolayers were then rinsed with saline or EBSS-GF at 4°C and extracted with 4°C 6% trichloroacetic acid (TCA). After refrigeration at 4°C for 1 hour, ATP was measured by either a luciferin-luciferase light-producing assay or by HPLC. For the luciferin-luciferase assay each plate was scraped with a rubber policeman, transferred to a 1.5 mL microcentrifuge tube and centrifuged at 1,750g for 5 minutes. Neutralizing the extracts before assaying did not change the measured ATP values and was not done. The buffered firefly lantern extract was incubated overnight in distilled water at 4°C. Before use

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the extract was centrifuged at 1,900g for 10 minutes and the
decanted supernatant mixed 2:1 with a buffer of sodium arsenate
(100 mmol/L) and magnesium chloride (40 mmol/L) to a total
volume of 25 mL. A standard stock solution of ATP (10 mmol/L)
mixed in 10% TCA was diluted to yield standards of 5 to 25 mmol/L
ATP. The reaction mixture consisted of 2 mL enzyme/buffer mixed
with 50 µL sample or ATP standard at room temperature. Lumines-
cence was measured 30 seconds after mixing from a continuous
recorder linked to an Aminco Fluoro-Colorimeter (American
Instrument Company, Silver Spring, MD).1

The HPLC assay of ATP used the method of Tekkanant et al.14
The adenine nucleotides were separated on an ion-pairing reversed
phase C18 column using a mobile phase of 60 mmol/L KH2PO4,
0.45 mmol/L tetrabutylammonium phosphate, and 3.6% acetoni-
trile (pH 3.2). Samples were precipitated with 6% TCA at 4°C,
neutralized with 1 volume 20% tri-n-octylamine in Freon 113; the
aqueous phase was then filtered through a 0.45 µmol/L nitrocellul-
ose filter. The pH was adjusted to 3.2 with phosphoric acid, 1.5 mL
samples were injected onto the column and eluted at a flow rate of 1
mL/minute using a Beckman high performance liquid chromatogra-
phy system (Beckman Instruments, San Ramon, CA). Peaks were
detected at 254 nm and quantitated by comparison with peak heights
of standards run on the same day.

Albumin flux measurements. Porcine pulmonary artery endo-
thelial cells were plated onto 6.5-mm-diameter Transwell polycarbon-
ate membranes (precoated with 30 µg/mL fibronectin for 30
minutes) with 0.4 µm pores (Costar, Cambridge, MA). Cells were
cultured in M199 with 10% FBS and used on day 3 after plating.
During the control period each monolayer was rinsed twice with
EBSS and then 150 µL of EBSS with 145 µmol/L albumin (cold);
then 33,000 cpm/mL of 14C albumin was added to the luminal chamber.
Each monolayer was then placed into the well of a 24-well tissue
culture plate containing 700 µL EBSS (these volumes balanced the
plate and 500 µL of the medium taken from the well and counted in
the scintillation counter. For the second 40-minute experimental
period, each monolayer was rinsed three times in EBSS-GF. One
day hundred fifty microliters of EBSS with or without 5.5 mmol/L
glucose, 25 mmol/L antimycin A and/or 12 mmol/L deoxyglucose
with 145 µmol/L albumin and 33,000 cpm/mL 14C albumin was
then added to the luminal compartment of each monolayer that was
placed into the well of a 24-well tissue culture plate containing 700
µL of the same solution without the albumin. After 40 minutes the
monolayer was removed and the lower chamber sampled as in the
control period. Albumin flux was calculated as follows:

\[
\text{counts through the monolayer} = \frac{cpm/pmole \times 40 \text{ min} \times 0.331 \text{ cm}^2}{
\text{ATP}}
\]

Dextran flux measurements. These data were determined in the
same manner as were the albumin flux measurements except that 1
mg/mL dextran (average molecular weight 67,900, 14.7 µmol/L)
and 145 µmol/L albumin were added to both sides of the monolay-
ers, and H2O2 dextran (average molecular weight 67,900, appro-
imately 235,000 cpm/mL) was added to the luminal side. In
addition, the monolayers were incubated with the metabolic inhibi-
tors for 15 minutes before initiating the measurement of the second
dextran flux. For the experiments in which monolayers were exposed
to H2O2, the dextran flux over a 40-minute control period was
measured, the monolayers were exposed to the indicated H2O2
concentration for 15 minutes, then treated with 10,000 units
catalase, washed, and a second 40-minute flux of dextran was
measured.

Statistical analysis. All data are presented as the mean ±
standard error unless otherwise indicated. Changes in albumin or
dextran flux were analyzed by analysis of variance, and differences
between groups were tested using a Tukey HSD post hoc test of
pairwise mean differences. Differences were considered significant at
the P < .05 level.

RESULTS

Effects of H2O2 on ATP levels in endothelial cells. We
previously reported that oxidants reversibly increased albumin
flux across porcine pulmonary artery endothelial cell
monolayers after a 15-minute exposure.5,13 When similar
endothelial cells were exposed to H2O2 concentrations be-
tween 100 µmol/L and 2.5 mmol/L, there was a significant
decrease in ATP after 15 minutes to approximately 25% of
control values (Fig 1). When catalase was added after a
15-minute exposure to 2.5 mmol/L H2O2, ATP levels were
23.1% ± 2.0% of control after 30 minutes (Fig 2). The
threshold concentration of H2O2 necessary to cause a
decrease in ATP was between 10 and 50 µmol/L (Fig 1). These
dose-response relationships are similar to those reported by
others.4 The degree of ATP depletion was similar between 50
µmol/L and 2.5 mmol/L H2O2 after 15 minutes.

Effects of metabolic inhibitors on ATP levels. If oxidant-
induced increases in macromolecule flux across endothelial
monolayers are caused by oxidant-induced ATP depletion,
then quantitatively similar depletion of ATP with metabolic
inhibitors would be expected to alter macromolecule flux
across the endothelial monolayers. To assess this, we first
measured the extent of ATP depletion caused by some
inhibitors of ATP production. Incubating endothelial cells in
glucose-free media caused a gradual, small decline in ATP
levels. At 20 minutes ATP levels were 92% ± 1.2% of control
and 77% ± 6.6% of control at 40 minutes in endothelial cells
incubated in glucose-free EBSS. Deoxyglucose (12 mmol/L), an inhibitor of glycolytic ATP production, caused a
significant decrease in ATP in the absence of glucose at 10,
20 and 40 minutes (Fig 3). When deoxyglucose was com-

Fig 1. ATP levels in porcine pulmonary artery endothelial cells
exposed to graded concentrations of H2O2 for 15 minutes. Results
are expressed as % of control value — 34.9 ± 2.5 pmoles ATP/µg
cell protein (n ≥ 3 for each value).

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bined with antimycin A, an inhibitor of mitochondrial ATP production, in the absence of glucose, ATP was depleted to 7% ± 3% of control within 10 minutes, and to 2% ± 1.3% of control within 20 minutes (Fig 3).

Effects of metabolic inhibitors on macromolecule flux.
We next evaluated the effects of metabolic inhibitors of ATP depletion on the flux of macromolecules across endothelial monolayers. None of the combinations of metabolic inhibitors caused a significant change in albumin flux across the endothelial monolayers (Fig 4). In particular the combination of antimycin A and deoxyglucose, which led to the most extensive ATP depletion, caused no change in the albumin flux.

Because there is controversy about how albumin crosses endothelium, we also measured the effect of the metabolic inhibitors on the flux of dextran across endothelial monolayers. Deoxyglucose caused a small but statistically significant decrease in the dextran flux. The change in the flux of dextran across monolayers treated with antimycin A and deoxyglucose was not different from the change in the flux across control monolayers (Fig 5).

In contrast to the lack of effect of metabolic inhibitor-induced reductions in ATP on transendothelial macromolecule flux, treatment of endothelial monolayers with 2.5 and 5.0 mmol/L H2O2 for 15 minutes increased the flux of dextran across the monolayers (Fig 6). ATP in monolayers from the same passage of cells and exposed to the same concentrations of H2O2 for 15 minutes were 6% ± 0% and 5% ± 0.6% of control at 15 minutes and 28% ± 0.2% and 25% ± 1.1%
ATP depletion and endothelial permeability

Dextran flux across porcine pulmonary artery endothelial cell monolayers during a 40-minute control period and then 40 minutes after a 15-minute exposure to Medium 199 or Medium 199 with 2.5 or 5.0 mmol/L H₂O₂. After the 15-minute exposure all monolayers were exposed to catalase (10,000 units), washed and the second 40-minute flux was measured. The increase in flux across monolayers exposed to 2.5 and 5.0 mmol/L H₂O₂ was greater than that across controls. The increase in flux across the monolayers exposed to 5.0 mmol/L H₂O₂ was greater than the increase in flux across monolayers exposed to 2.5 mmol/L H₂O₂.

of control at 60 minutes, for 2.5 and 5.0 mmol/L H₂O₂ respectively.

**DISCUSSION**

Oxidants cause ATP depletion in cultured endothelial cells. They increase the permeability of cultured endothelial monolayers to macromolecules and the permeability of an intact microvasculature to ions. However, the relationship between ATP depletion and the changes in permeability have previously not been directly addressed. Hinshaw et al found that ATP depletion, via glucose deprivation, to 10% of control levels over 3 hours resulted in progressive shortening and aggregation of microfilaments accompanied by retraction and rounding in P388 murine lymphoma cells. On the other hand, Wysolmerski found that a similar degree of ATP depletion in endothelial cells treated with antimycin A and deoxyglucose caused only subtle and infrequent changes in cell-to-cell contacts and prevented cell retraction in response to histamine, cytochalasin B and ethchlorvynol. Holmsen et al observed that platelets depleted of ATP to approximately 25% of control with H₂O₂ retained a normal shape-change response when exposed to ADP. More recently Hinshaw showed that endothelial cells depleted of ATP to less than 5% of control levels with metabolic inhibitors underwent a shape change only after subsequent exposure to the ionophore A23187. However, ATP depletion by itself did not change endothelial cell shape; similar changes in endothelial cell shape after exposing endothelial cells to A23187 have been reported with A23187 alone, without ATP depletion. Thus the role of ATP in the cytoskeletal control of cell shape is complex and may vary among cell types.

In this investigation we found that depletion of ATP with metabolic inhibitors, to <5% of control levels, did not increase albumin or dextran flux across endothelial cell monolayers over 40 minutes. This is a time course similar to that used in our current and prior experiments when we observed that oxidants reversibly increased albumin flux across similar monolayers. It is also similar to the time course for the reversible increase in ionic conductance of cerebral microvessels exposed to oxidants, as reported by Olesen. It is possible that if such severe ATP depletion had been extended for several hours, the macromolecule flux might have changed, although it is not clear that this would have represented a reversible effect.

The degree of ATP depletion seen with antimycin A and deoxyglucose in the absence of glucose (<5% of control) was as great or greater than that seen with oxidant doses capable of increasing the flux of albumin across the endothelium. Xanthine and xanthine oxidase (10 mU/mL) increased the flux of albumin across endothelial monolayers after a 15-minute exposure. ATP levels in endothelial monolayers exposed to 10 mM/L xanthine oxidase have been reported to be greater than 50% of control after 30 minutes and 1 hour. Similarly, 10 mM/L glucose oxidase increased endothelial monolayer permeability to albumin. In the same study, the same doses of glucose oxidase decreased endothelial ATP to approximately 75% of control levels. Exposing endothelial monolayers to 2.5 mmol/L H₂O₂ for 15 minutes increased both transendothelial albumin flux, as well as the transendothelial dextran flux in the current investigation. However, endothelial cell ATP was greater after 2.5 mmol/L H₂O₂ than after antimycin A and deoxyglucose. Because antimycin A and deoxyglucose treatment did not increase transendothelial macromolecule flux, oxidant-induced ATP depletion alone does not explain the increase in transendothelial macromolecule transfer seen after endothelial exposure to oxidants.

In related experiments Bolin et al found that inhibition of glycolysis in an isolated perfused rabbit lung model did not alter the hydraulic conductivity of the lung. Oxidative ATP production was not blocked, nor were ATP levels measured in Bolin's study, so the level of ATP depletion achieved is not known. However, Bolin's observations are consistent with our own finding that inhibition of glycolysis with deoxyglucose did not increase macromolecule flux across the endothelium. Rasio et al found that the combination of iodoacetic acid (IAA), potassium cyanide and hypoxia increased capillary permeability in the eel swimbladder. However, these agents have effects independent of ATP depletion that may contribute to changes in capillary permeability. IAA inhibits glycolysis by acetylating sulphydryl groups in glyceraldehyde phosphate dehydrogenase. However, IAA reacts with any susceptible sulphydryl groups and has been shown to cause increases in vascular permeability independent of its effects.
on ATP. In addition, hypoxia has been demonstrated to increase capillary permeability by the generation of free radicals.

Although ATP depletion alone does not increase macromolecule flux across endothelial monolayers, ATP depletion may contribute to the oxidant-induced changes in permeability. Oxidants cause phospholipase C hydrolysis of phosphatidylinositol in endothelial cells. ATP depletion has been demonstrated to potentiate the phospholipolysis that occurs after exposing cells to phospholipase C, and oxidant ATP depletion may potentiate oxidant-stimulated phospholipase C activity.

In summary, we do not find that ATP depletion alone can explain oxidant-induced changes in the endothelial barrier to macromolecules. However, oxidants cause simultaneous changes in many cell functions. ATP depletion may act in concert with other pathways to alter endothelial barrier function.

REFERENCES

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