Intestinal Ischemia–Reperfusion Injury Causes Pulmonary Endothelial Cell ATP Depletion

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Intestinal ischemia–reperfusion is a common clinical event associated with both clinical and experimental distant organ injury. In particular, the pulmonary microvasculature appears to be susceptible to injury resulting from systemic inflammatory mediator activation. This study was designed to evaluate the hypothesis that noncellular humoral factors associated with intestinal ischemia–reperfusion result in pulmonary endothelial cell adenosine triphosphate (ATP) depletion. Male Sprague-Dawley rats had intestinal ischemia induced by microvascular clip occlusion of the superior mesenteric artery (SMA) for 120 minutes. Reperfusion resulted from superior mesenteric artery clip removal. After reperfusion for 0, 15, or 30 minutes, plasma samples were obtained from the portal vein. Monolayers of cultured rat pulmonary artery endothelial cells then were incubated with the plasma samples. Adenosine triphosphate levels were determined using a luciferin-luciferase assay. A ^{51}Cr-release assay using labeled endothelial cells was performed under identical conditions to assess cytotoxicity. Potential mechanisms of ATP depletion were evaluated by analysis of cellular energy charge and assessment of microfilament architecture. Endothelial cell ATP levels decreased from $2.23 \pm 0.16 \times 10^{-11}$ moles/μg DNA in sham preparations to $1.23 \pm 0.09 \times 10^{-11}$ moles/μg DNA ($p < 0.001$) after 4 hours in plasma from animals undergoing 120 minutes of intestinal ischemia. For plasma obtained after 15 minutes of reperfusion, the decrease in cellular ATP concentration persisted ($1.23 \pm 0.27 \times 10^{-11}$ moles/μg DNA, $p < 0.001$ *vs.* sham). After 30 minutes' reperfusion, cellular ATP levels increased only slightly after the 4-hour incubation ($1.39 \pm 0.26 \times 10^{-11}$ moles/μg DNA, $p < 0.005$ *vs.* sham). No significant cytotoxic injury occurred in any group when compared with controls. Cellular energy charge was unchanged, and microfilament architecture was preserved. These data confirm the hypothesis that humoral factors, independent of the neutrophil, result in endothelial cell ATP depletion without metabolic inhibition or cell death. Depletion of energy stores by noncellular humoral factors may represent an early event that predisposes the cell to more severe injury by other mediators of the endogenous inflammatory response.

Multiple organ failure, or MOF, is a leading cause of death in surgical and posttrauma patients in the intensive care unit.[1] Clinically, MOF may be defined as a progressive deterioration in the ability of the visceral organs to perform normal physiologic functions. The pathogenic events that initiate MOF include many common clinical conditions. Thermal injury,[2,3] sepsis,[1,4,5] hemorrhagic shock,[6,7] trauma,[8] acute pancreatitis,[9,10] and ischemia–reperfusion events[11–13] are all known precipitants

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of MOF. Each stimulus is apparently unique; however, all are associated with activation of the endogenous inflammatory response. Some of the inflammatory mediators appear to be part of a common pathway resulting in distant organ injury.

Acute lung injury associated with the MOF syndrome is a subject of particular interest and clinical importance.[10,11,14] A substantial portion of the morbidity and mortality associated with MOF is directly attributable to the adult respiratory distress syndrome (ARDS).[15] Adult respiratory distress syndrome is associated with an overall mortality rate higher than 50% in the context of MOF[15,16] and may account for 100,000 deaths annually in the United States.[17] Therapeutic interventions have been directed primarily toward the symptoms of pulmonary dysfunction, because the underlying pathogenic mechanisms of this clinical entity are poorly understood. Current evidence supports the concept that activation of a systemic inflammatory process leads to "targeting" of the alveolar endothelial cell, acute lung injury, and clinical respiratory failure.

An ARDS-like acute lung injury induced by an ischemia–reperfusion injury to the rat intestine has been described previously.[11] This experimental lung injury was characterized by histologic evidence of alveolar capillary endothelial cell injury, increased microvascular permeability, reduced lung tissue adenosine triphosphate (ATP) levels, and pulmonary sequestration of neutrophils. The current study was designed to evaluate the hypothesis that humoral factors, independent of the cellular constituents of blood (primarily the neutrophil), mediate pulmonary endothelial cell injury in this clinically relevant setting. Because the *in vivo* preparation does not allow for independent manipulation of the intestinal and pulmonary injuries, an *in vitro* pulmonary endothelial cell preparation was developed. A preliminary assessment of this combined model of pulmonary endothelial cell injury after an *in vivo* MOF stimulus and also of the pathogenic mechanisms leading to endothelial cell injury is provided.

METHODS

Animal Model

Pathogen-free male Sprague-Dawley rats (150–200 g, Charles River Laboratory Inc., Portage, MI) were used for all experiments. All experimental protocols were approved by the University of Michigan Committee on Use and Care of Laboratory Animals. Animals were fasted for 12 hours immediately before experiments. Anesthesia was administered by intramuscular injection of 100 mg/kg ketamine hydrochloride (Aveco Co. Inc., Fort Dodge, IA). Midline laparotomy was performed, and the superior mesenteric artery was occluded by application of a noncrushing microvascular clip. Reperfusion was achieved by removal of the microvascular clip at a second laparotomy after a second anesthetic (50 mg/kg intramuscular ketamine hydrochloride). Intestinal ischemia was maintained by superior mesenteric artery occlusion for 120 minutes, followed by microvascular clip removal and reperfusion for 0, 15, or 30 minutes. This procedure provided a profound reversible reduction in intestinal blood flow confirmed by laser–Doppler and microsphere analysis.[11,18] In addition, relative hemodynamic stability was maintained during the experimental periods.[11] Sham-operated control animals underwent identical preparation except that the superior mesenteric artery clip was not applied (no ischemic injury resulted). Blood samples were obtained from the portal vein after desired ischemia and reperfusion time and after 180 minutes in sham-operated animals. Death was by exsanguination after the portal venous phlebotomy. Samples were drawn into cold heparin-coated plastic syringes and pooled for each experimental group. The pooled blood samples then were centrifuged at $3000g \times 15$ minutes at 3 C. Plasma from each sample was decanted into cold sterile plastic conicals and held on ice until use.

Endothelial Cell Preparation

The rat pulmonary artery endothelial cell line was provided by U. Ryan; the isolation and characterization of these cells has been described previously.[19] Cells were maintained in culture using minimal essential medium of Eagle with Earle's balanced salt solution (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 10% fetal bovine serum, 0.1 mmol/L nonessential amino acids (Gibco Laboratories Inc., Grand Island, NY), 2.0 mmol/L L-glutamine (Irvine Scientific, Santa Ana, CA), penicillin G 100 U/mL, streptomycin sulfate 100 μg/mL, and fungizone 0.25 μg/mL. The cells were grown at 37 C in a humidified 5% CO_2 atmosphere. Cells were subcultured by trypsinization when confluent monolayers were obtained.

Cellular ATP Determination

Endothelial cells were seeded into a 24-well culture plate in 1 mL/well culture media. Cells were incubated until near-confluent monolayers were achieved (80% to 90% confluency) representing approximately 2×10^5 cells/well. Each monolayer then was washed twice with Dulbecco's modified Eagle medium (DMEM, Gibco Laboratories Inc., Grand Island, NY). Plasma from each experimental ischemia–reperfusion group, as well as the sham group, then was diluted with DMEM to 25% (vol/vol) and 1.0 mL placed onto the endothelial cell mono-

layers. The 24-well plate then was incubated at 37 C in a humidified 5% CO_2 atmosphere for 4 hours. At the conclusion of the incubation period, the entire 24-well plate was snap-frozen in a liquid nitrogen bath and stored at −70 C.

At the time of assay, the 24-well plate was slowly warmed to the melting point and then held on ice. Each individual well was sonicated for 15 seconds to lift and disrupt any intact or adherent endothelial cells (Kontes, Micro Ultrasonic Cell Disrupter, Model ASI). Cellular ATP levels were determined by the method of Stanley and Williams[20] as adapted for use with endothelial cells[21,22] with modifications as described. Luciferin-luciferase in glycine salt (Sigma Chemical Co., St. Louis, MO) was prepared 30 minutes before use by mixing with sterile H_2O to a final concentration of 4.0 mg/mL. A standard stock solution of 10 mmol/L adenosine 5′-triphosphate (ATP) disodium salt (Sigma Chemical Co., St. Louis, MO) was prepared in phosphate buffer (10 mmol/L KH_2PO_4, 4 mmol/L $MgSO_4$, pH 7.75) and stored at −70 C. Eight standard curve solutions were prepared by serially diluting this stock solution for an [ATP] range of 10^{-4} to 10^{-9} mol/L. Using a dry heating block, 1.5-mL aliquots of the phosphate buffer were dispensed into test tubes and preheated to 100 C. Aliquots of 250 μL were taken of standards and samples and added to the boiling phosphate buffer. After vortexing, the solutions were heated for 5 minutes at 100 C and then placed on ice. To eliminate any artifactual enzymatic ATP hydrolysis during the assay, 1.0 mL of each sample or standard solution was mixed with 1.5 mL arsenic buffer (10 mmol/L Na_2HASO_4, 4 mmol/L $MgSO_4$, pH 7.75). A 500-μL aliquot of the samples or standards in arsenic buffer then was transferred to a luminometer cuvette. Luminescence intensity was measured by integration for 10 seconds after a 2-second delay on addition of 100 μL of the luciferin-luciferase reagent using an LKB-Wallac 1251 Luminometer and 1291 Dispenser (LKB-Wallac, Turku, Finland).

DNA Determination

After sampling of the 24-well plate for ATP determination, DNA analysis was carried out using the method of Labarca and Paigen[23] as modified below. Hoechst 33258 reagent (Sigma Chemical Co., St. Louis, MO) was prepared by diluting in phosphate-buffered saline (pH 7.4) to a final concentration of 20 μg/mL. A standard stock solution of highly polymerized calf thymus DNA sodium salt (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 1.0 mg DNA in 10.0 mL phosphate-buffered saline. Standard curve solutions were prepared by serially diluting the stock solution to a range of concentrations from 5 − 100 μg/mL. The final assay solution consisted of 100 μL standard solution or sample (directly from 24-well plate), 100 μL Hoechst 33258 reagent solution, and 1.8 mL DNA buffer (50 mmol/L NaH_2PO_4, 50 mmol/L Na_2HPO_4, 2.0 mol/L NaCl, pH 7.4). After vigorous vortexing, fluorescence was measured at 356 nm excitation and 458 nm emission on a Perkin-Elmer LS-5B Fluorimeter.

High-performance Liquid Chromatography Adenine Nucleotide Analysis

Experiments for high-performance liquid chromatography analysis were performed with rat pulmonary artery endothelial cell line in monolayers of 6×10^6 cells. Cells were incubated in 25% plasma samples under conditions identical to those for ATP determinations. At the conclusion of the 4-hour incubation period, nucleotides were extracted by scraping cells into 80% methanol (Mallinckrodt Inc., Paris, KY) heated to 75 C as described by Shryock et al.[24] Samples were centrifuged, evaporated to dryness, reconstituted in distilled H_2O, and filtered through a Millipore type HA micropartition system (Millipore Corp., Bedford, MA) to remove insoluble material. Nucleotides then were separated by the method of Tekkanat and Fox,[25] with modifications as noted. Standard curves were generated for ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inosine-5′-monophosphate (IMP) daily. Nucleotides were separated on a C-18 reverse-phase column (Waters μBondapak C-18, 3.9 × 300 mm). Separation was by isocratic elution using the following reagents: 0.86% acetonitrile/water, 1 mmol/L tetrabutylammonium phosphate (pairing agent), and 65 mmol/L KH_2PO_4 at a flow rate of 1.2 mL/minute, pH adjusted to 2.9. A Waters 712 WISP injector in conjunction with a Waters 484 Tunable Absorbance Detector (Waters Assoc., Milford, MA) set at 254 nm was used for all measurements.

Fluorescent Staining of Microfilaments

Endothelial cells were grown in 6-well plates to an approximate density of 1 to 2×10^5 cells / cm^2. Cells were incubated in 25% plasma for 4 hours as in the ATP determination experiments. Staining of adherent endothelial cells with fluorescent phallotoxin[26,27] was performed as follows. Cells were fixed with 2% paraformaldehyde at 25 C and held on ice for 1 hour. After washing, the cells were permeabilized with 0.2% Triton X-100 for 5 minutes. After a second washing, 165 nmol/L rhodamine phalloidin was added to the monolayer and incubated for 20 minutes at room temperature in the dark (to prevent photobleaching). After another wash step, a glass

coverslip was sealed to the monolayer with a drop (5 μL) of 90% glycerol. Stained samples then were viewed with a Nikon optiphot fluorescence microscope. Fluorescence micrographs at either 400× or 1000× magnification were taken, using Tmax film (Eastman Kodak, Rochester, NY).

Cytotoxicity Assay

Cytotoxicity was measured using a ^{51}Cr-release assay.[28] Endothelial cells were seeded into a 24-well culture plate. Each well received 2 μCi $Na^{51}CrO_4$ (New England Nuclear, Boston, MA). The plate was incubated for 24 hours until monolayers reached near-confluency (approximately 2×10^5 cells/well). Immediately before use, monolayers were washed twice with DMEM to remove unincorporated radioactivity. Cells then were incubated in 25% plasma as described for ATP determinations. After a 4-hour incubation period, culture media was removed from each well and centrifuged at 3000$g \times 5$ minutes. The supernatants (0.5 mL) were aspirated and analyzed on a gamma counter (Auto-Gamma 5000, Packard Instrument Co., Downers Grove, IL). Spontaneous release was measured in wells receiving DMEM only (controls). Maximal release was obtained in wells receiving 0.2% Triton X-100. Spontaneous release measured less than 10% in all experiments. Cytotoxicity then was calculated using the following formula:

$$\% \text{ Cytotoxicity} = (CPM_{experimental} - CPM_{control}/ CPM_{maximal} - CPM_{control}) \times 100\%$$

Statistical Methods

All data are expressed as the mean plus or minus the standard error of the mean. Statistical analysis was performed using analysis of variance and a post hoc comparison among groups with Fisher's protected least squares difference test (Statview II, Abacus Concepts Inc., Calabasas, CA). Significance was assigned when the F test for multi-comparison was significant and $p < 0.05$ was achieved.

RESULTS

This animal model of intestinal ischemia and reperfusion yields a time-dependent, progressive intestinal injury that provides a consistent stimulus for MOF. The acute pulmonary microvascular injury was previously characterized *in vivo*.[11]

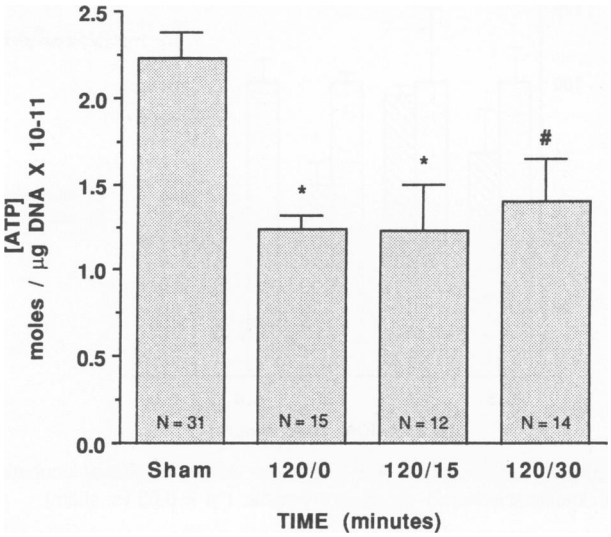

Figure 1. Endothelial cell ATP levels after 4-hour incubation with intestinal ischemia–reperfusion plasma. Data are pooled from five individual experiments with sample size indicated. Time points on the x-axis represent minutes of intestinal ischemia/minutes of intestinal reperfusion; for example, 120/0 represents 120 minutes of ischemia and no reperfusion. This nomenclature is used in all subsequent figures. (*$p < 0.001$ *vs.* sham; #$p < 0.005$ *vs.* sham).

Cellular Adenosine Triphosphate Depletion by Ischemia–Reperfusion Plasma

Measurement of endothelial cell ATP levels after incubation with intestinal ischemia–reperfusion plasma was performed based on the hypothesis that depletion of cellular energy stores is consistent with cellular injury or metabolic stress. As shown in Figure 1, endothelial cells exposed to portal venous plasma from injured animals demonstrated a significant decrease in cellular ATP levels. Cells incubated in plasma from sham-operated animals had a stable ATP level of $2.23 \pm 0.16 \times 10^{-11}$ moles/μg DNA. Incubation with plasma from animals subjected to 120 minutes of ischemia, however, resulted in a significant decrease in endothelial cell ATP content to $1.23 \pm 0.09 \times 10^{-11}$ moles/μg DNA ($p < 0.001$). Cells incubated with plasma obtained after 15 and 30 minutes of reperfusion contained $1.23 \pm 0.27 \times 10^{-11}$ moles ATP/μg DNA ($p < 0.001$ *vs.* sham) and $1.39 \pm 0.26 \times 10^{-11}$ moles ATP/μg DNA ($p < 0.005$ *vs.* sham), respectively. Data shown are endothelial cell ATP levels after 4 hours of incubation with ischemia–reperfusion plasmas. A significant decrease in cellular ATP content was noted as early as 2 hours and persisted for at least 8 hours of incubation (Fig. 2).

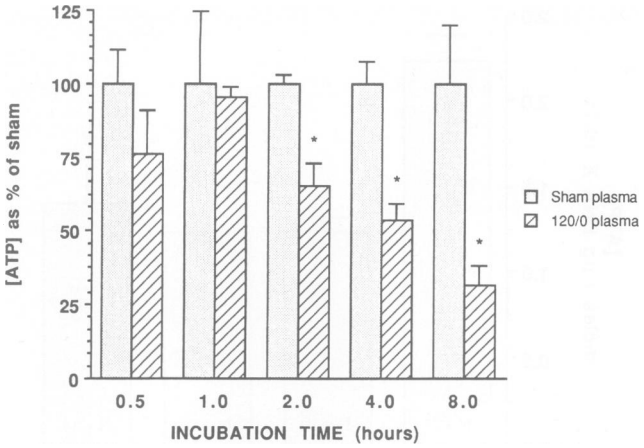

Figure 2. Endothelial cell ATP levels after various lengths of incubation with intestinal ischemia–reperfusion plasma. (*$p < 0.05$ *vs.* sham).

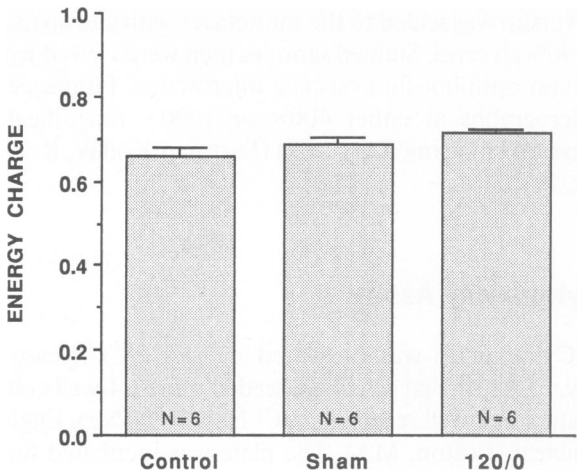

Figure 4. Cellular energy charge as calculated for control, sham, and injured experimental groups. No significant change resulted from 4-hour incubation with plasma from animals sustaining intestinal ischemia–reperfusion injuries.

Stability of Cellular Energy Charge

Analysis of nucleotide pools in the endothelial cells was carried out by high-performance liquid chromatography as above. Three experimental groups were analyzed: a control group consisting of cells incubated in DMEM growth medium only, a sham group consisting of cells incubated in 25% plasma from sham-operated animals, and an injured group consisting of cells incubated in 25% plasma from animals sustaining 120 minutes of intestinal ischemia. As shown in Figure 3, nucleotide pools remained relatively constant between the three groups. Only insignificant changes were noted in the relative levels of ADP, AMP, and IMP when compared with ATP levels (ATP normalized to 100% in each group for comparison purposes). Thus there appeared to be no accumulation of ATP precursors despite the marked fall in overall cellular ATP levels.

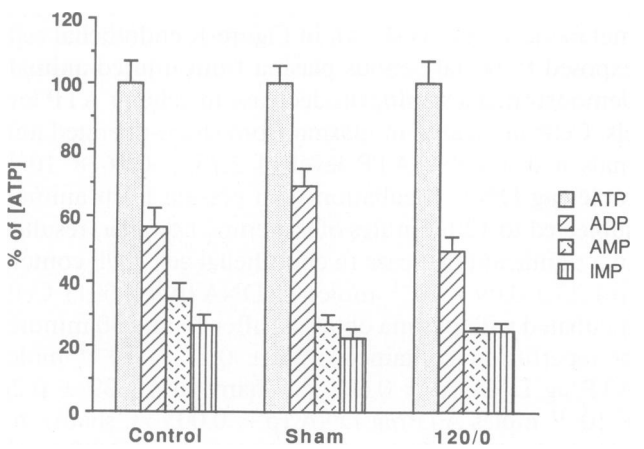

Figure 3. Endothelial cell nucleotide pools after 4 hours' incubation with either normal growth medium (control), plasma from sham-operated animals (sham), or intestinal ischemia–reperfusion plasma (120/0). Data standardized for comparison purposes by defining ATP concentrations as 100% in each group.

Under normal conditions, cellular energy levels are carefully maintained by balancing energy-consuming and energy-producing reactions. Energy charge is a ratio of the intracellular adenylate pools:

$$\text{Energy Charge} = (\text{ATP} + \tfrac{1}{2}\,\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$$

Cellular metabolism appears to be tightly regulated by alterations in energy charge.[29] Inhibition of metabolic pathways leads to profound decreases in cellular energy charge. Energy charge levels below 0.5 are associated with loss of membrane integrity and cell viability. Energy charge was calculated for each of the above experimental groups, and the results are depicted in Figure 4. No significant difference existed between groups, suggesting that the mechanism of cellular ATP depletion is not metabolic inhibition.

Preservation of Cytoskeletal Architecture

Further insight into the mechanism of cellular ATP depletion was provided by an analysis of microfilament architecture using the fluorescent phallotoxin staining technique. Endothelial cells subjected to metabolic inhibition and subsequent ATP depletion characteristically demonstrate disrupted or fragmented microfilaments.[22,27] Three experimental groups were analyzed: control cells in normal growth media, sham cells in uninjured plasma, and injured cells in plasma from animals sustaining ischemic injury. Normally, microfilaments exist as fine linear stress fibers traversing the cyto-

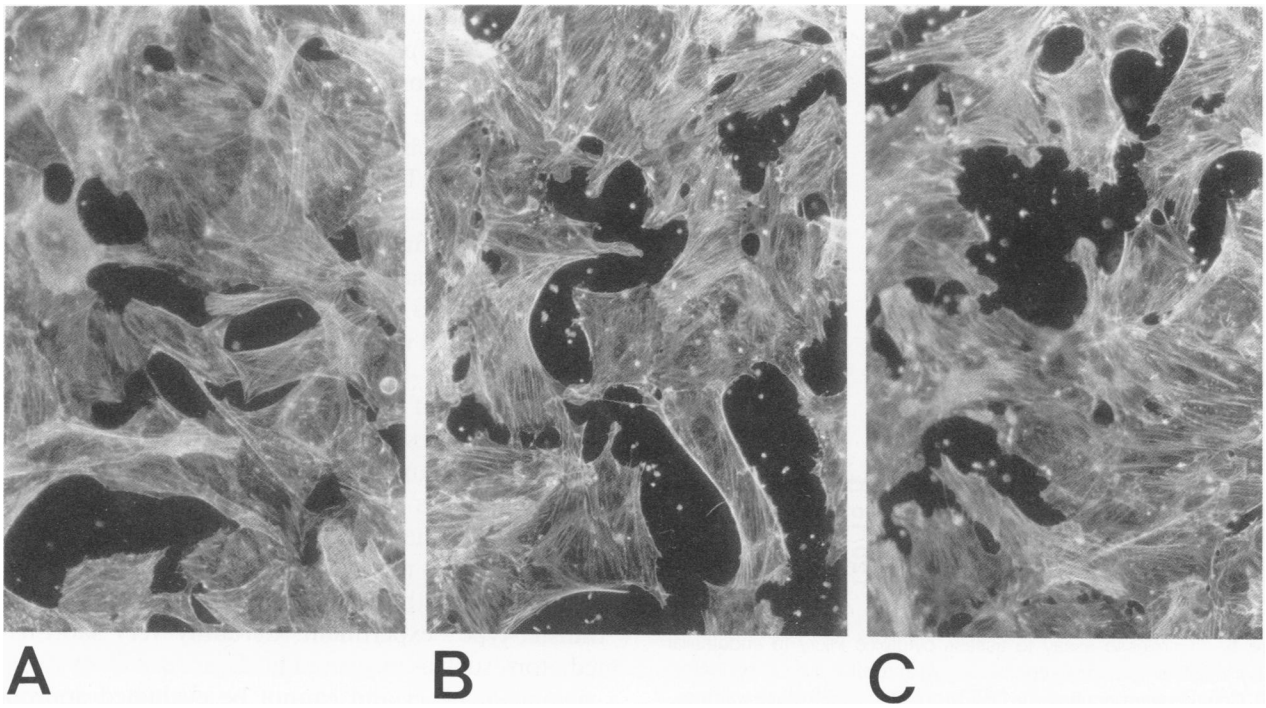

Figure 5. Endothelial cells after fluorescent staining with rhodamine phalloidin. (A, left) Control group consisting of cells incubated 4 hours in normal growth medium. Note the fine linear stress fibers traversing the cytoplasm and concentrated at the cell periphery. (B, center) Cells incubated 4 hours with plasma from sham-operated animals. Microfilament architecture is unchanged. (C, right) Cells after 4 hours in plasma obtained from the portal vein after 120 minutes' intestinal ischemia (resulted in 45% decrease in cellular ATP levels). Again, microfilaments remain intact and are essentially unchanged from controls (magnification ×400 on Kodak Tmax film).

plasm and concentrated at the periphery of the cell. As can be seen in Figure 5 a through c, this pattern persists under all three experimental conditions. Despite significant decreases in cellular ATP levels, microfilament architecture was preserved, suggesting that the mechanism of ATP depletion may be one of enhanced metabolic activity with consumption of energy stores and not one of metabolic inhibition.

Cytotoxic Injury to Endothelial Cells

The ^{51}Cr-release assay is a reliable and quantitative method of analyzing cytotoxic injury to endothelial cells resulting in membrane disruption.[30] In view of the observation that endothelial cell ATP levels were significantly depleted by ischemia–reperfusion plasmas yet the energy charge maintained, the issue of whether the injury was cytotoxic was assessed. As shown in Figure 6, no significant cytotoxicity resulted from incubation of endothelial cells with plasma from either sham or injured animals as compared with normal growth medium controls. Hydrogen peroxide exposure at a concentration of 500 nmol/mL served as an internal control and consistently produced a cytotoxic injury of 85% ± 2.5%.

DISCUSSION

This model of an *in vivo* stimulus of MOF combined with an *in vitro* preparation of cultured pulmonary endothelial cells provides a straightforward, reproducible, and clinically relevant format in which to study the pathogenic mechanisms associated with distant organ injury and ARDS. Important characteristics of the experimental model are: (1) it is a consistent, reproducible stimulus of MOF; (2) the determinants of endothelial cell injury can be assessed independently from the intestinal injury; and (3) the role of cellular elements (specifically the neutrophil) in the pathogenesis of acute lung injury may be studied both separately and in conjunction with humoral factors.

Vascular endothelium appears to be a critical early target in the pathogenesis of distant organ injury associated with activation of the inflammatory system. Intact endothelial cell metabolic and functional activities are required to maintain the vascular compartment.[31] Loss of the integrity of the endothelial monolayer has been implicated as a primary event in the development of acute edema in various vascular beds, particularly the lung.[32,33] Evidence continues to accumulate that the pul-

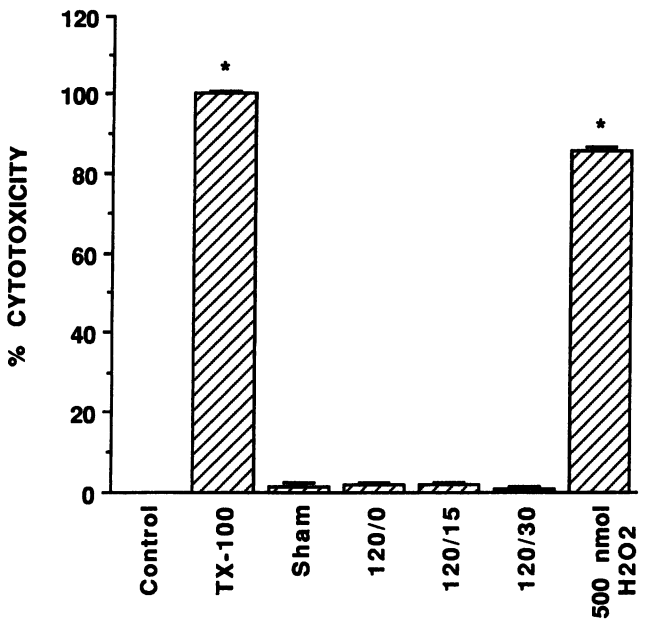

Figure 6. ^{51}Cr-release assay to assess cytotoxic injury to endothelial cells by intestinal ischemia–reperfusion plasma after 4-hour incubation period. Controls were incubated in DMEM only. Maximal release was measured with Triton X-100. H_2O_2 injury served as internal control. (*$p < 0.001$ *vs.* control).

monary endothelial cell is a primary and particularly vulnerable site for injury initiated by many different clinical events and mediated by cytokines, neutrophils, proteases, toxic oxygen metabolites, and other factors.[8,15,34–37] The model described in this study was designed to evaluate endothelial cell injury by humoral inflammatory mediators in the context of MOF. The pulmonary microvascular injury may be the critical step in the development of ARDS.

Humoral factors associated with intestinal ischemia–reperfusion injury result in depletion of pulmonary endothelial cell energy stores. As noted in other studies,[32,38,39] depletion of cellular ATP levels serves as an early marker of endothelial cell dysfunction. Whether the cell consumes ATP at an accelerated rate to maintain integral cell functions (such as stabilization of membranes) in response to exposure to I/R plasma constituents is yet to be demonstrated. Maintenance of energy charge in cells exposed to injured plasma, however, argues that metabolic inhibition of ATP synthetic pathways is not the underlying mechanism of the observed cellular energy store depletion. Further evidence against a metabolic inhibitory mechanism is provided by the assessment of microfilament architecture. Metabolic inhibition of ATP synthetic pathways has been shown to cause disruption of microfilaments in the face of significant ATP depletion.[22,27] In the current study, microfilament architecture was preserved despite a 45% fall in ATP levels. Accelerated consumption exceeding synthetic capacity appears to be a more likely mechanism for depletion of cellular energy stores in this setting. Further studies to define the precise humoral factors responsible for the aforementioned observations are ongoing.

Depletion of ATP in the vascular endothelium as an isolated event appears unlikely to be sufficient to lead to the devastating clinical scenario of ARDS. As shown by the ^{51}Cr-release data, humoral factors do not result in cytotoxic injury to the endothelial monolayer. Other mediators associated with the inflammatory system and intestinal ischemia–reperfusion injury are likely to promote the injury of the already compromised endothelial cell. Although these additional mediators have not been characterized, candidates include the neutrophil,[40,41] various cytokines (tumor necrosis factor, platelet-activating factor, others),[35,42] endotoxin,[43–45] proteases,[34,46] and toxic oxygen metabolites.[37,47]

Limitations of the model are inherent in its design as a "transfer-type" experiment. Certainly very short-lived mediators, such as oxygen radicals, are not likely to have a significant effect and cannot be evaluated appropriately. Oxygen radicals generated in the gut and released into the portal circulation, however, are unlikely to contribute to the *in vivo* pulmonary injury as observed in our model. The neutrophil–endothelium microenvironment is the more likely circumstance for injury mediated by oxygen radicals.

In addition to the cellular injury hypothesis, an alternative explanation for the depletion of energy stores in endothelial cells after intestinal ischemia–reperfusion should be considered. Activation of vascular endothelial cells has been shown to occur both *in vivo* and *in vitro* in association with inflammation and with a variety of inflammatory mediators.[48] Activated endothelial cells appear to play a role in enhanced adhesion of neutrophils, lymphocytes, and monocytes to vascular endothelium after exposure to various cytokines. Interleukin-1, tumor necrosis factor, and lymphotoxin all have been shown to increase endothelial cell adhesiveness for leukocytes and to modulate procoagulant activity.[49] Studies suggest that the mechanism of adhesion involves expression of cell surface adhesion molecules such as ELAM-1, MEL-14 antigen, and H-CAM.[48,49] The process is time dependent, requiring protein and RNA synthesis. As demonstrated by Bevilacqua and Gimbrone,[49] the time from activation of endothelial cells (activated with tumor necrosis factor or interleukin-1) to peak cell surface adhesion molecule expression, neutrophil binding, and peak procoagulant activity is 4 hours.[49] In view of the time course of ATP depletion observed in the current study, the potential association between endothelial cell activation and depletion of cellular energy stores deserves further investigation.

In summary, these studies describe a combined *in vivo/in vitro* model designed for the investigation of the pathogenesis of acute pulmonary injury after a stimulus of MOF. Humoral factors, independent of the neutrophil, result in endothelial cell ATP depletion without metabolic inhibition or cell death. Depletion of energy stores by noncellular humoral factors may represent the early event that predisposes the cell to more severe injury by other mediators of the endogenous inflammatory response.

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