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In Vitro Modeling of Non-Hypoxic Cold Ischemia-Reperfusion Simulating Lung Transplantation

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Abstract

Objective—Although anoxia/reoxygenation of cultured cells has been employed to model lung ischemia-reperfusion injury (IRI), this does not accurately mimic events experienced by lung cells while a lung is retrieved from a donor, stored, and transplanted. We developed an *in vitro* model of non-hypoxic IRI to simulate these events.

Methods—Human umbilical vein endothelial cells (HUVECs) underwent simulated cold ischemia by replacing 37°C culture media with 4°C Perfadex™ solution for 5 hours in 100% O_2. Culture dishes were allowed to warm to room temperature for 1 hour (implantation), then Perfadex™ was replaced with 37°C culture media (reperfusion).

Results—During cold ischemia, HUVEC filamentous actin cytoskeleton quickly became rearranged, and gaps developed in the previously confluent monolayer occupying 20% of the surface area. Simulated reperfusion resulted in reorganization to a confluent monolayer. Development of gaps was not due to enhanced necrosis based on LDH retention assay. Endothelial cytoskeletal rearrangement could account for early edema due to IRI with reperfusion. MAPK and NF-κB activation occurred with simulated reperfusion despite normoxia. Pro-inflammatory cytokines IL-6 and IL-8 were significantly elevated in media at the end of reperfusion.

Conclusions—Exposing HUVECs to simulated cold ischemia without hypoxia causes reversible cytoskeletal alterations, activation of inflammatory pathways, and elaboration of cytokines. Because this model accurately depicts events occurring during lung transplantation, it will be useful to explore mechanisms regulating lung cell response to this unique form of IRI.

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Introduction

Lung transplantation (LTX) is appropriate for patients with end-stage lung disease. However, transplant success is limited by a shortage of suitable donor lungs, early post-transplant lung dysfunction, and later bronchiolitis obliterans syndrome (BOS). A potential solution to the lung donor shortage is the use of lungs recovered from non-heart-beating donors (NHBDs – patients who have died suddenly of cardiac arrest, outside the hospital or in the emergency room),[1–3] but this strategy is associated with development of pulmonary edema and impaired gas exchange in recipient animals.[4] Early graft failure is a major cause of morbidity and the leading cause of early mortality following LTX.[5] Early graft dysfunction ia a risk for development of BOS.[6] Thus, improved understanding of lung ischemia-reperfusion injury (IRI) would benefit recipients of conventional LTX, facilitate transplantation of lungs recovered from NHBDs, and might reduce the risk of BOS.

IRI is a coordinated series of events involving components of the innate and adaptive immune systems.[7] A hallmark of lung IRI is pulmonary edema due to increased permeability of endothelial cells[8] associated with inflammation and elaboration of pro-inflammatory cytokines. The cellular and molecular mechanisms responsible for the phenotype of the injury remain unknown.

The goal of our study was to develop an *in vitro* cell culture model of IRI that realistically mimics the sequence of events experienced by human endothelial cells during lung retrieval, cold storage and transplant.

Materials and Methods

Cell Culture

HUVECs (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were seeded at a density of at 2500 cells/cm^2 on collagen-coated (Invitrogen, San Diego, CA) 100 mm diameter cell culture dishes, maintained in Clonetics™ EGM-2 BulletKits® medium (Cambrex), containing 1.8 % fetal bovine serum, growth factors, heparin, hydrocortisone and gentamicin/amphotericin B at 37°C in a humidified incubator in 95% room air/5% CO_2 and grown to confluence. Cells were subcultured on similar dishes at 2500 cells/cm^2 after enzymatic digestion with 0.1% trypsin in 1mM EDTA for 5 minutes at 37° C and used between the third and tenth passage.

Model of simulated IRI

HUVECs on 35 mm diameter glass bottom dishes with integral coverslips (MatTek Corp, Ashland, MA) grown until 100% confluent were placed inside a sealed Plexiglas chamber modified to simulate the rapid changes that occur during the course of lung recovery, storage, implantation and reperfusion, depicted in Figutre 1A. Cell culture medium (37°C) was suddenly replaced with cold Perfadex™ (Vitrolife, Kungsbacka, Sweden) buffered with Tham® (Abbott Pharmaceuticals, Chicago, IL) to pH 7.2, and the Plexiglas chamber was maintained at 4°C on ice and the box ventilated with 100% O_2 at 1 l/min. It took 1 hour for temperature to reach 4°C after replacement of media with Perfadex™. Following 4 additional hours of simulated cold ischemia, the Plexiglas chamber was allowed to warm to room temperature for 1 hour to simulate implantation. To simulate reperfusion, Perfadex™ was suddenly replaced with 37°C culture media and the Plexiglas chamber was ventilated with 95% room air/5% CO_2, in a 37°C oven. Dishes were removed from the chamber in triplicate at different time points throughout the experiment, and immediately fixed in 4% paraformaldehyde. Cells maintained in EGM2- medium at 37°C in humidified 5% CO_2 incubator were used as “24 hour controls”; medium was changed at the same timepoints when

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medium or Perfadex™ was changed in experimental dishes. The time line of the experiment is depicted in Figure 1B. Probes inserted through sealed ports continuously recorded temperature, pH and PO2 in one representative cell culture dish on a pc by PicoRecorded Software (Pico, St Neots, UK).

Phalloidin Staining and Image Analysis

HUVECs were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed 3 times with PBS. Dishes were stored at 4°C and stained the following day for F-actin filaments using Alexa Fluor® 568 phalloidin (Invitrogen, OR). Cells were incubated for 1 hour at room temperature in the dark with 1/100 dilution of phalloidin in 1% BSA/PBS/0.05% Tween 20, then rinsed three times with PBS. Coverslips were immediately examined with Leica DMIRB Inverted Fluorescence/DIC microscope at 200X and 400X magnification and confocal microscope at 400X, and 3 photographs taken to evaluate cell shape and F-actin cytoskeleton. Gap surface area was quantified on gray scale images with MetaMorph™ software (Universal Imaging Corp, Downingtown, PA). Each cell was labeled by a masked observer as "normal" or "abnormal" with respect to actin filaments. "Normal" cells had actin fibers at the periphery and crossing the cell, whereas "abnormal"cells had no central actin filaments - actin was either not apparent or visualized only in the cell periphery. Labeled cells were quantified by Metamorph™ software. Features of the actin cytoskeleton were evaluated in groups by a masked observer (KB - an actin cytoskeletal expert).

Cell Viability Assay

In separate experiments at different time points during simulated IRI, cells and cell culture media were assessed for lactate dehydrogenase (LDH) activity using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) per manufacturer's instructions. Cell viability was expressed by cytoplasmic LDH as a percent of total LDH. Culture medium and Perfadex™ were used as background controls to normalize the absorbance value.

Protein extraction and Immunoblotting

At various time points, HUVECs cultured on 60 mm collagen-coated culture dishes until 100% confluent were subjected to simulated IRI, and protein was extracted and Western blots performed as previously described.[9] Monoclonal antibody to phosphorylated p38 was from BD Bioscience Pharmigen. Polyclonal antibody to p38 and monoclonal β-actin antibodies were from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies to JNK1/2/3, ERK1/2, and IκBα were from Cell Signaling Technology (Danvers, MA). Secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). Immobilon Western Chemiluminescence HRP substrate was from Millipore (Billerica, MA). Films were scanned on an Epson Precision 4180 flatbed scanner at 600 dpi in 16-bit grayscale with the Epson Scan software and saved in TIFF format. The densities of bands obtained from the same membrane were measured using the 1-D Gel Scan function in the MetaMorph™ Software package and expressed as relative optical density to a blank lane loaded with lysis sample buffer. The ratio of phospho/total for MAPKs and IκBα/β-actin at each time point on each blot was normalized to the corresponding ratio of Controls on each blot by dividing each value by the Control ratio. Thus, the ratios of Controls for each blot were arbitrarily set to one. Expression at each time point was compared to Control 24 hour samples, cells that were maintained at 37°C in an incubator with media changed at the same times as media was changed in experimental dishes.

IL-6 and IL-8 measurements

After 24 hours of "reperfusion", cell culture media was collected from samples in triplicate, briefly centrifuged and stored at −20°C before IL-6 and IL-8 were measured by ELISA

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according to manufacturer's instructions (DuoSet ELISA Development kit, R&D Systems, Inc., Minneapolis, MN).

Statistical Analysis

All data are reported as mean ± SEM. Statistical analysis was performed using Statistica (Statsoft, Tulsa, OK). ANOVA with Tukey's honest significant difference for post hoc corrections, or Student's t test was used for data analysis. Because of large variances that were proportional to means, normalized intensity data of Western blots was $\log_{10}$-transformed.[10] A p value <0.05 was considered significant.

Results

The cell culture model reflects the environment of lung tissue during IRI

The experimental plan is detailed in Figure 1, which shows that acidosis occurs, but hypoxia is not a feature of the model.

Simulated cold non-hypoxic ischemia causes actin cytoskeletal rearrangement and formation of gaps in the monolayer

HUVECs maintained inside the incubator at 37°C and ventilated with 95% O_2/5% CO_2 were 100% confluent and showed the typical cobblestone appearance. The filamentous actin network in untreated HUVECs consisted of peripheral and cell-spanning stress fibers. Replacement of culture medium with Perfadex™ at 4°C resulted in rearrangement of the actin cytoskeleton, with most of the HUVECs demonstrating disappearance of F-actin stress fibers and/or peripheral location of the stress fibers (Figure 2). Using an actin cytoskeleton normality index, nearly 60% of cells were observed to be abnormal within 15 minutes of the onset of cold ischemia. F-actin rearrangement persisted through the early phase of reperfusion (15 minutes), but appeared normal after 4 hours of reperfusion, when actin cytoskeleton normality index returned to pre-IRI levels (Figure 3A). An observer masked to group identity noted dramatic changes in the actin stress fibers during cold ischemia and early after reperfusion (6 photos/group, 3 at 200X, 3 at 400X, selected randomly), with return to a normal appearance following four hours of reperfusion (Table 1).

Cytoskeletal alterations were associated with formation of gaps in the confluent monolayer. After 15 minutes and 4 hours at 4°C (simulated cold ischemia) gaps were obvious and accounted for ~20% of the surface area (Figure 3B). Following 1 hour of simulated re-warming (when Plexiglas boxes were removed from the ice and allowed to rewarm to room temperature simulating implantation time), gap area was reduced to ~4% surface area although actin filaments were still abnormal. Following simulated reperfusion (when Perfadex™ was replaced with 37°C cell culture medium) the gaps became smaller (~3.5%), and after 1 hour of reperfusion, gap area was normal, and actin stress fibers began to reassemble with complete reorganization of the monolayer (100% confluent) after 4 hours reperfusion. This was maintained through 24 hours post-reperfusion (Figure 3A).

To ensure that gap formation was not a result of cell death or loss of cellular adherence, we measured cell viability at various times throughout cold ischemia, rewarming, and reperfusion. Cell viability was maintained at ~90% for all times assessed during simulated IRI out to 3 hours reperfusion (Figure 3C) despite cytoskeletal alteration and formation of gaps indicating that cold ischemia-induced gap formation was not due to cell death or detachment. Detached cells would be in the supernatant and lysed, resulting in increased LDH levels. Interestingly, after 24 hours of simulated reperfusion, cell viability decreased to ~82% of the 24 hour control, suggesting either that IRI might limit the proliferative capacity of HUVECs relative to the 24

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hour control, or that delayed cell death, perhaps due to apoptosis, was occurring as a consequence of simulated cold IRI.

Simulated IRI causes MAPK activation and IκBα degradation

Simulated IRI was associated with activation of MAPKs (ERK 1/2, SAPK/JNK 1/2, p38) and NF-κB pathways (Figure 4), almost exclusively during reperfusion. p38 was activated weakly during simulated cold ischemia, more during warming before reperfusion, with maximal phosphorylation after 5 and 15 minutes of simulated reperfusion. Baseline phospho-ERK was present in control HUVECs (in media, no manipulation), which is not unexpected since the media was not growth factor depleted.[9] ERK and JNK activation was dramatic after 5 minutes of simulated reperfusion. IκBα degradation was evident after 5 minutes of simulated reperfusion. Activation of NF-κB is controlled by inducible phosphorylation and degradation of the inhibitory proteins IκB (IκBα, IκBβ, IκBε) that bind and inhibit activation of NF-κB by masking its nuclear localization sequence. Thus, degradation of IκBα indicates NF-κB activation. Because of slightly reduced intensity and variability in 24 hour Control samples, these reductions in IκBα/β-actin were not statistically significant. Although p38 activation begins to some extent during simulated cold storage and warming, the major stimulus for MAPK and NF-κB activation in our model is simulated reperfusion. Taken together, this data suggests that MAPK and NF-κB activation is preceded by, and not the cause of, endothelial intercellular gap formation.

Simulated IRI results in elaboration of pro-inflammatory cytokines IL-6 and IL-8

Because MAPK and NF-κB activation are commonly implicated in transcription of certain pro-inflammatory chemokines and cytokines, we measured IL-6 and IL-8 in the media of HUVECs after simulated IRI. IL-6 and IL-8 were significantly elevated 24 hours after reperfusion (Figure 5).

Discussion

Our model of simulated IRI exposed HUVECs to an environment similar to that experienced by lung endothelial cells during recovery for transplant and implantation into a recipient. Although some investigators have employed hypoxia/reoxygenation to model IRI in cell culture,[11, 12] this does not accurately simulate the environment experienced by lung cells during transplantation. Conventional organ donors are almost always ventilated with 100% oxygen. In rats breathing room air before sacrifice, we showed that pO_2 did not fall below 70 mm Hg in lungs maintained at 37°C for 4 hours although many of the cells died, presumably from substrate depletion and acidosis.[13] During cold storage, aerobic metabolism persists, presumably because of availability of alveolar oxygen.[14]

Replacing cell culture media with buffered cold Perfadex™, a commonly used pulmonary preservation solution, causes rapid and sustained changes in endothelial cell actin cytoskeleton associated with formation of gaps in the monolayer. The endothelial cytoskeleton, particularly F-actin stress fibers, is responsible for maintaining cell shape and connections between cells, and plays a critical role in regulation of pulmonary vascular permeability.[15] The cytoskeleton may also function as an intracellular communication system or signaling scaffold. Although these changes begin to reverse with rewarming (simulating lung implantation), endothelial monolayers were still abnormal at the time of simulated reperfusion. Attachment of endothelial cells to the basement membrane of alveolar capillaries *in vivo* may limit movement more than attachment to collagen-coated culture dishes *in vitro*. Nevertheless, if this *in vitro* phenotype reflects changes that occur *in vivo*, then it is not surprising that early pulmonary edema is a feature of lung IRI. This may also explain why controlled reperfusion (the practice of restricting flow to a lung graft for the first 5 minutes) results in better gas exchange.[16] Controlled

reperfusion may result in less edema as gaps in the monolayer resolve in the early minutes following reperfusion. Indeed, our data suggest that 5 minutes may not be enough controlled reperfusion time. Delaying impantation of the second lung longer than 15 minues may not be practical, but could be an area for future study. Our model may uncover a strategy to reduce endothelial ultrastructural abnormalities during ischemia that might be clinically tested.

We simulated reperfusion by sudden replcement of Perfadex™ with warm cell culture medium. *In vivo*, Perfadex™ in the lung vasculature warms slowly during lung implantation, then is suddenly replaced with warm blood resulting in an abrupt change in temperature and pH. Our simulated reperfusion caused activation of MAPKs and NF-κB associated with elaboration of inflammatory cytokines. Although there was some activation of p38 during cold ischemia and warming, simulated reperfusion was responsible for most of the activation of signaling pathways we studied, although actin cytoskeletal re-arrangement and gap formation occurred during simulated ischemia. It is possible that MAPK and NF-κB activation may play a role in re-establishment of a normal cytoskeleton and closure of intercellular gaps, but this process begins during rewarming before substantial pathway activation. Thus, we suspect the two processes are unrelated. We measured inflammatory cytokines only after 24 hours reperfusion. In this *in vitro* system, the cytokines are stable; additional studies to document the time course of cytokine production would be useful.

This pattern of signaling pathway activation is identical to that seen in whole lung tissue following warm lung IRI in mice and conventional lung transplantation in rats (unpublished ongoing work in our laboratory). Although we did not assess cell proliferation or apoptosis, apoptosis may account for our observed late increase in cell death assessed by LDH assay (Figure 3C). Apoptosis occurs following lung transplantation[17] and is associated with activation of p38 and JNK MAPKs,[18] but the clinical significance of apoptosis is unknown.

Our study complements a study by Hall et al[19], who showed similar actin cytoskeletal derangements in cultured porcine pulmonary artery endothelial cells cooled to 4°C for 2 or 4 hours, and similar gaps in the monolayer, but these were not quantified. Endothelial cell shape recovered quickly with rewarming but actin stress fibers were still poorly organized one hour after re-warming. In another study, the impact of different organ preservation solutions was assessed, and although there were some differences, cooling alone by replacing the cell culture media with media at 4°C had considerable impact on actin cytoskeleton.[20]

Cardella et al subjected human pulmonary epithelial cancer cells (A549 cells) to simulated IRI using a similar model, replacing cell culture media with cold low potassium dextran-glucose solution or variations of this solution. In their model, prolonged ischemia (12 hours or longer) led to increasing amounts of epithelial cell death and separation from the culture plate, worsened by simulated reperfusion.[21] Inoue et al used A549 cells to show that NF-κB activation in cells cooled to 4°C occurred after rewarming, and IL-8 mRNA levels were increased one hour after rewarming.[22] This is consistent with our observation that simulated reperfusion appears to be the main stimulus for NF-κB and MAPK activation, not the antecedent period of hypothermia. However, malignant cells may not be ideal to study, and bronchial epithelial cells *in vivo* may not contact preservation solution flushed through the vasculature, so the relevance of these models to LTX is unclear.

MAPKs are activated in cell culture in response to a variety of stimuli, including mechanical stretch[18] and TNF-α or stimulation of Nod-like and toll-like receptors (TLRs).[23] Recently, we showed that TLR4 on pulmonary parenchymal cells, *not* bone marrow dericed macrophages, mediates early edema in a murine model of *in situ* normothermic lung IRI,[24, 25] Why replacing Perfadex™ after a period of hypothermia with 37°C cell culture medium should stimulate signaling pathways associated with inflammation is unclear, and is an area for future study.

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However, because activation occurs primarily with reperfusion, it may be practical to provide inhibitors of some of these signalling pathways either to the donor before organ recovery, or in the preservation solution.

There is no doubt that other cell types, such as alveolar macrophages (AMs), participate in lung IRI,[26] but we have shown that TLR4 on parenchymal cells plays a more important role in early edema formation due to IRI than TLR4 on AMs using chimeric TLR4 mice.[25] Accordingly, we chose to establish our model with endothelial cells. Furthermore, although AMs clearly contribute to MAPK activation that occurs with IRI, and some have argued that the AM is a "key coordinator" in the pathophysiology of lung IRI,[27] our data clearly show that endothelial cells also activate MAPKs, and elaborate inflammatory cytokines due to IRI in the absence of AMs. In addition, this study suggests a mechanism for IRI-induced edema independent of AMs, and offers an explanation for the observation that the practice of controlled reperfusion results in less edema.

This study validates a realistic model of simulated IRI that can be used to expose a variety of pulmonary cell types to simulated IRI and investigate a number of interventions to reduce IRI, such as inhibitors of MAPK or NF-κB activation. Limitations of the model include the absence of mechanical sheer stress and hydraulic pressure across the cell layer. Endothelial cell attachment to the extracellular matrix *in vivo* may be more robust than to collagen coated Petri dishes. Although our model suggests a role for endothelial cells as major mediators of edema due to IRI, impaired alveolar fluid clearance by alveolar epithelial cells may also contribute.[28] A potential criticism is our use of HUVECs instead of human pulmonary microvascular endothelial cells (HMVECs). We chose to create our model with HUVECs because they are inexpensive, readily available, well-characterized, and have been used to demonstrate the deleterious effects of pulmonary preservation storage solutions.[29] Now that we have a well-characterized model of simulated IRI, we plan to use it to study response of HMVECs, and alveolar Type II, and bronchial epithelial cells in air-liquid interface cultures to simulated IRI. In preliminary experiments with HMVECs, similar changes in actin rearrangement and gap formation occur. There is no doubt that *in vivo*, different cell populations interact with each other and the extracellular matrix in response to stresses like IRI. The role of intravascular thrombosis and coagulation is ignored in *in vitro* cell culture models, as well as the contribution of leukocytes and AMs.

In conclusion, exposing human endothelial cells to simulated cold ischemia without hypoxia causes reversible cytoskeletal alterations associated with significant gaps in the otherwise confluent monolayer, and activation of inflammatory signaling pathways. Because this model accurately depicts events occurring during lung recovery and transplantation, it may be a valuable tool to explore mechanisms regulating endothelial cell response to IRI and therapeutic interventions.

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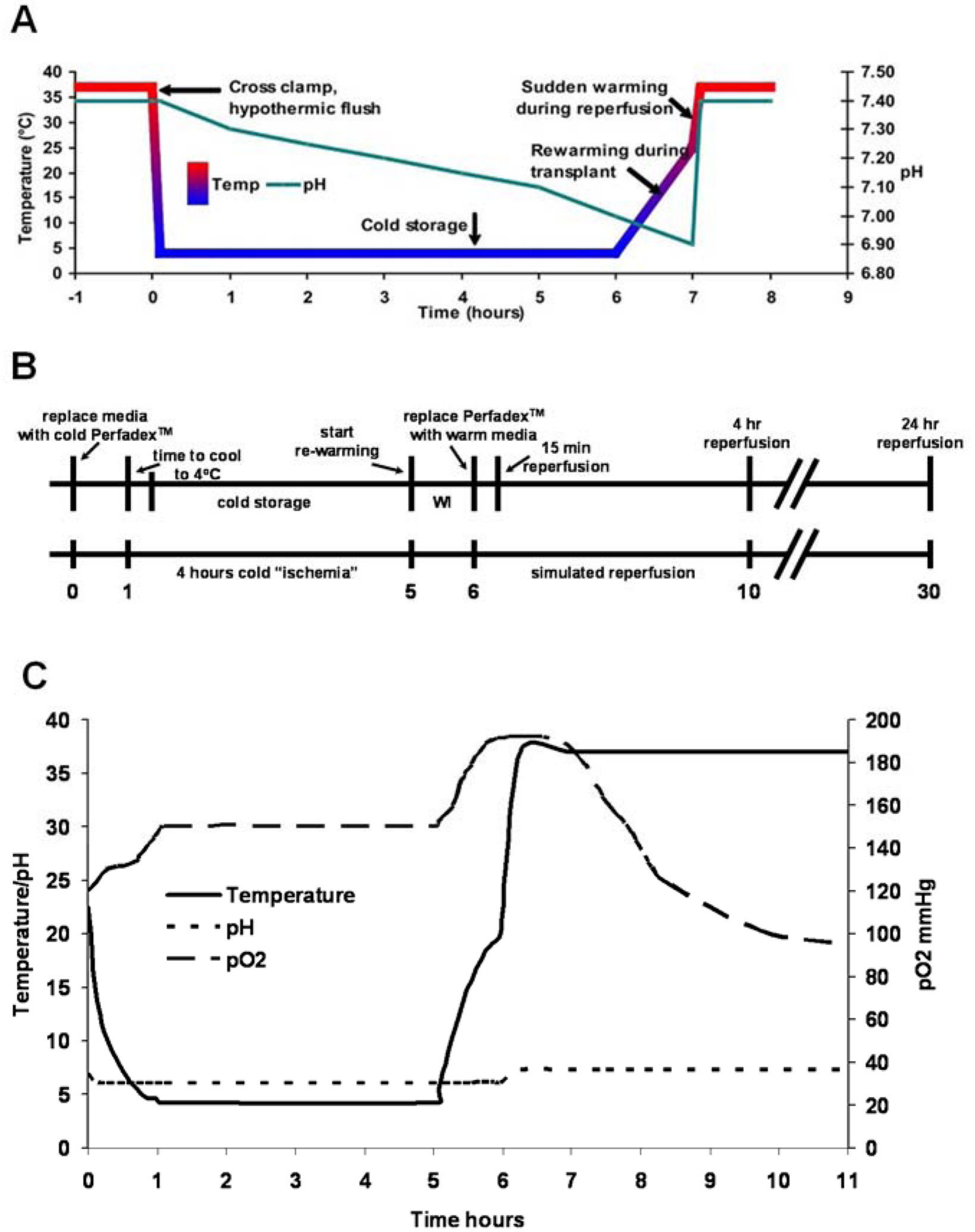

Figure 1.

A. Time course of events that occur during LTX modeled by our non-hypoxic cell culture model. Warm blood is suddenly replaced with cold preservation solution (Perfadex™) flushed through the pulmonary artery after cross clamping to cool the lung to 4°C. At the time of circulatory arrest, lungs are still ventilated with 100% oxygen and the trachea is clamped with lungs fully inflated with oxygen. After several hours of cold storage, the lung gradually rewarms to room temperature while the anastomoses are performed in the recipient (approximate time 1 hour). Then suddenly, circulation is restored and temperature is rapidly increased to 37°C. Figure from [13].

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B. Time course of experiment. In preliminary experiments, despite the replacement of warm media with cold Perfadex™, it takes approximately 1 hour for culture dishes in Plexiglas boxes to reach 4°C.

C. Data from a representative experiment showing measured change in temperature, pH and pO2 during simulated cold ischemia-reperfusion.

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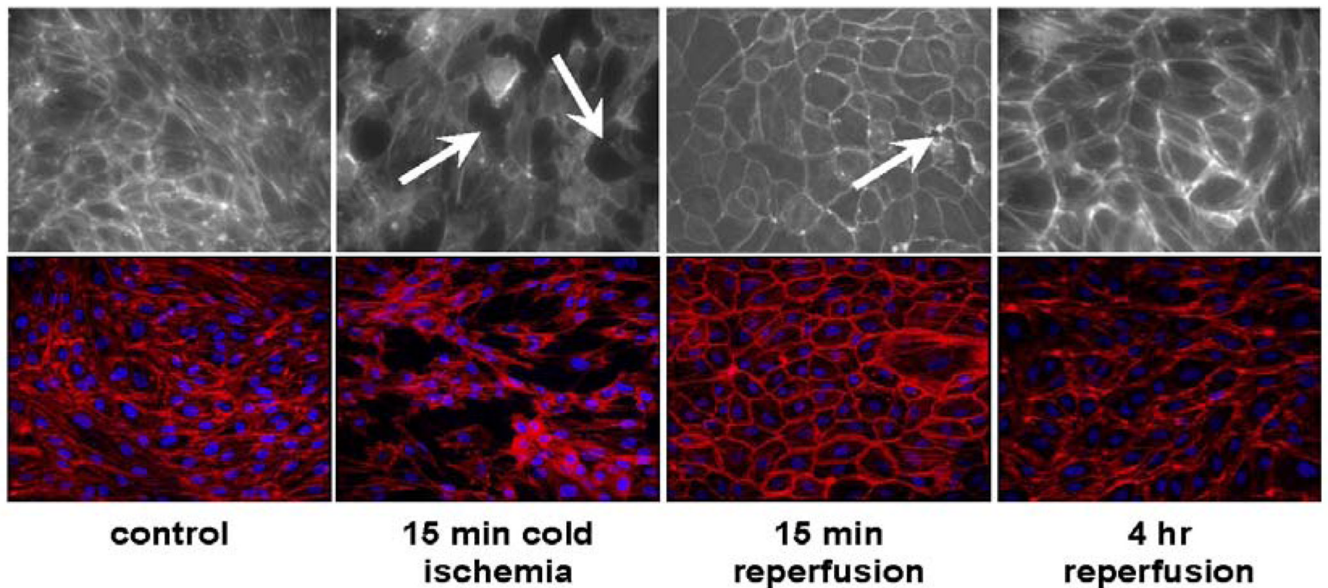

Figure 2.

Control cells maintained in media at 37°C were 100% confluent, actin stress fibers were evident in the cytoskeleton with focal adhesion between cells. After 15 minutes of cold “ischemia” at 4°C (replacement of warm media with cold Perfadex®), HUVECs quickly shrank loosing their junctions and disrupting the confluent monolayer with disassembly of actin filaments. This resulted in the formation of gaps in the monolayer (white arrows). After 4 hours of cold storage, monolayers looked the same (not shown). Early after simulated reperfusion, when Perfadex™ was replaced with media at 37°C, most of the stress fibers were not apparent, and actin was visualized at the periphery of cells, leaving only fragments of cortical actin. After four hours of reperfusion, actin filaments appeared to reassemble with reorganization of the actin network. Fluorescence (top) and confocal microscope images (bottom).

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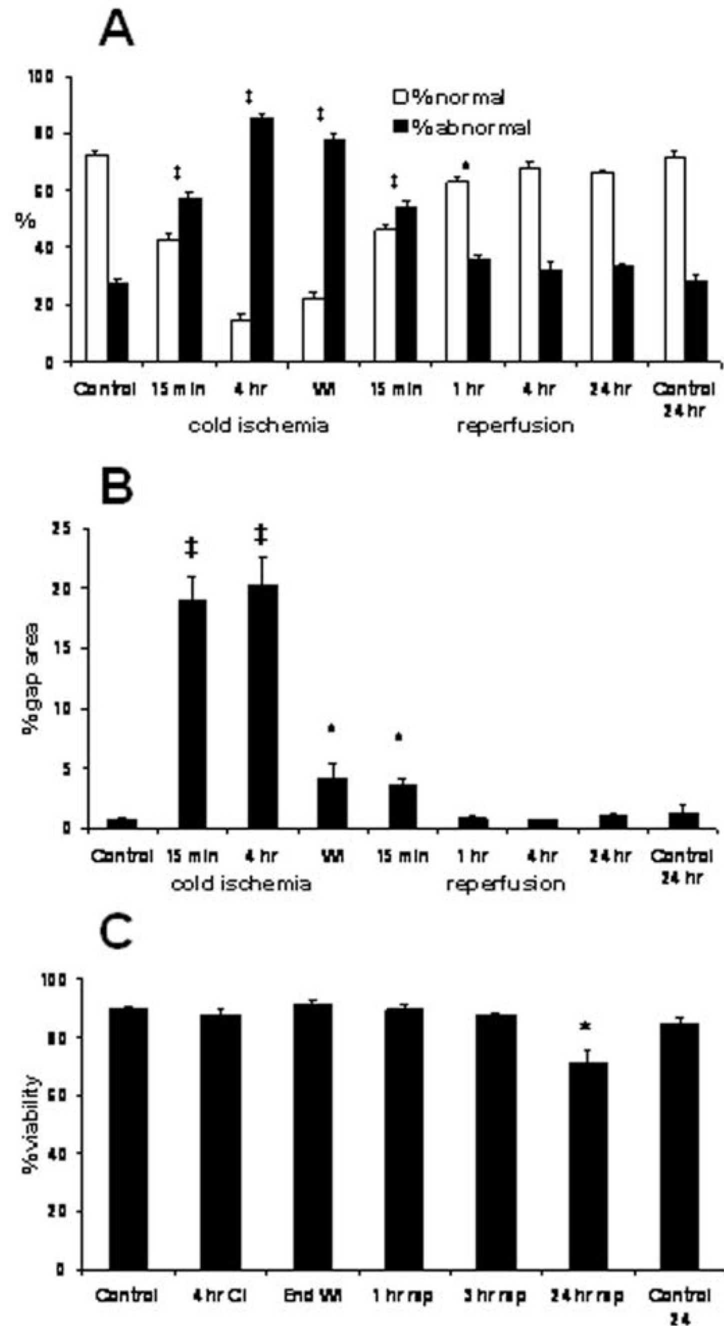

Figure 3.

A. Actin stress fibers disappear or are oriented peripherally (abnormal) in HUVECs exposed to simulated IRI. Cytoskeleton of each cell was graded by a masked observer as normal or abnormal. ‡ $p<0.001$, *$p<0.05$ ANOVA with Tukey's post hoc correction.

B. Area of gaps (%) in the monolayer of HUVECs subjected to simulated cold ischemia without hypoxia. Even though there is considerable recovery of the monolayer during one hour of warming (WI), there are still significant gaps at the onset of reperfusion and during early reperfusion, which resolve within one hour. ‡ $p<0.001$, ANOVA with Tukey's post hoc correction. Because of profound differences during cold ischemia, gap surface area at WI and

15 min rep do not reach significance with Tukey's post hoc correction. However *p<0.01 compared to Control by unpaired t test.

C. Viability is maintained in HUVEC monolayers despite gap formation. Thus, gap formation is not due to cell death or shedding. Late death (24 hr reperfusion) may be due to apoptosis. *p<0.05 ANOVA with Tukey's post hoc correction.

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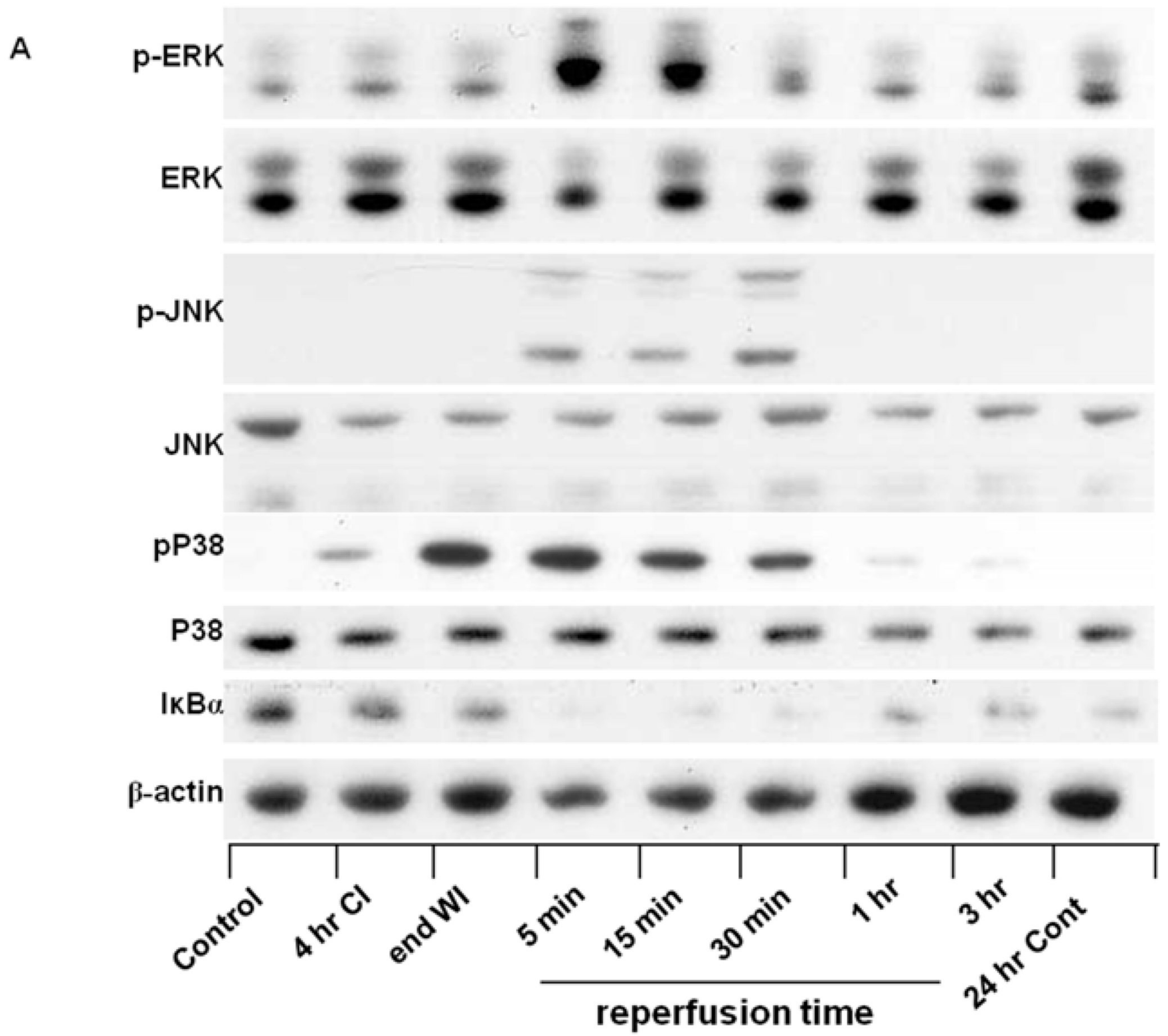

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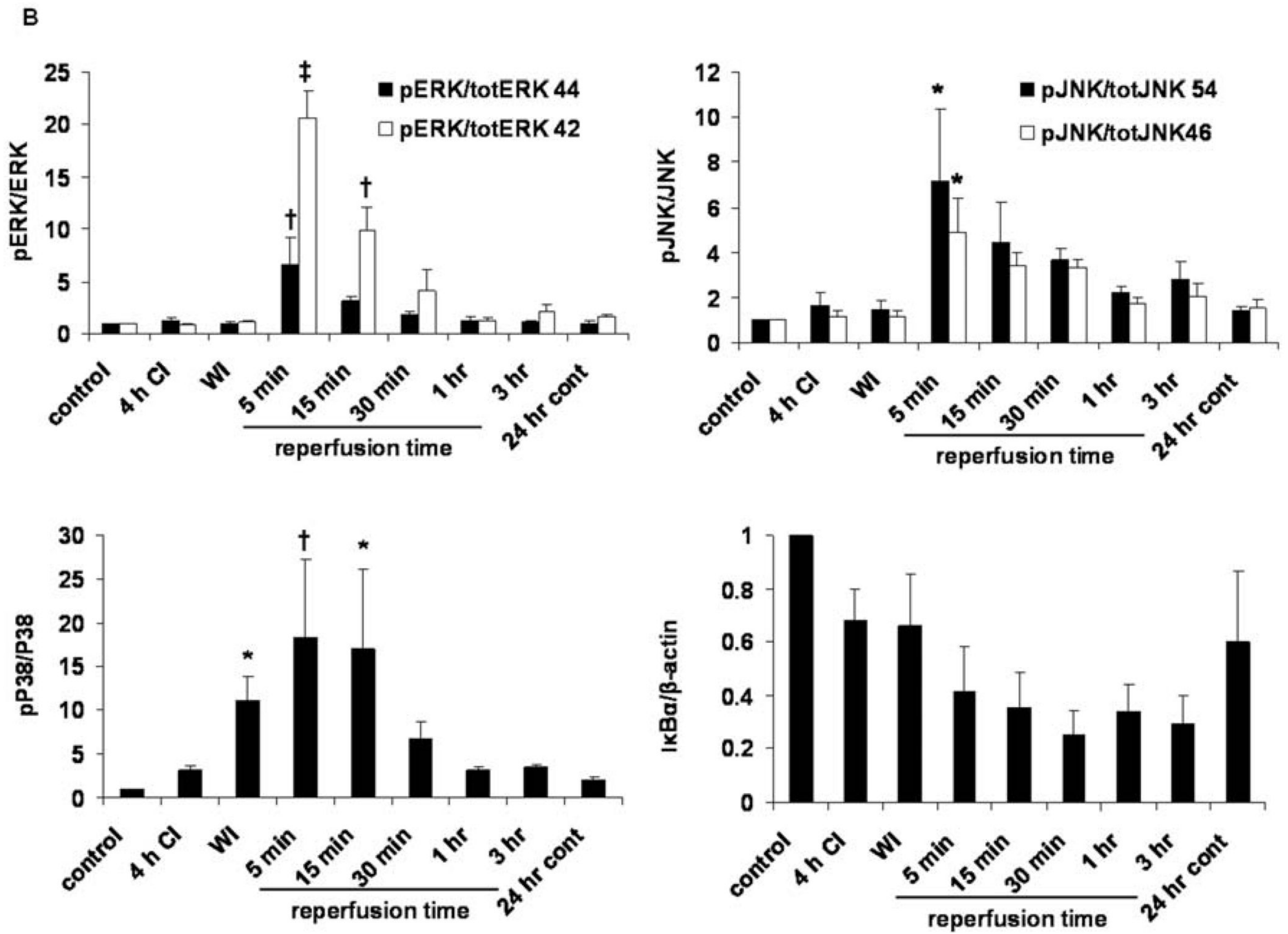

Figure 4.

A. Representative Western blots show some phosphorylation of ERK present in all samples, but increased phosphorylation after reperfusion. JNK activation is apparent with reperfusion only. p38 activation begins to become apparent during cold ischemia, increases during warming, and is most intense following reperfusion. IκBα degradation is apparent following reperfusion. Representative of triplicate experiments.

B. To compare expression of phospho/total, or IκBα/β-actin, ratios of intensity by laser scanning of controls was normalized to 1. Phosphorylation of ERK and JNK ocurs with simulated reperfusion. p38 activation begins to become apparent during cold ischemia and is significant with warming, and maximal following reperfusion. IκBα degradation occurs following reperfusion. Ratios of controls were arbitrarily set to 1 and all other ratios on the same blots adjusted so intensities of 3 experiments could be compared across time points, so comparison to controls could not be made. Control 24 hour specimens weere used to establish iif differences in intensity were significant. Because of large variances that were proportional to the means, data was $\log_{10}$-transformed. For MAPKs, ratios of phospho/total for Control 24 hour samples was similar to Controls. For IκBα/β-actin Control 24 hour intensities were less than Controls with more variability, so decreased IκBα following reperfusion obvious on all blots was not statistically significant. *$p<0.05$, † $p<0.01$, ‡ $p<0.001$ by ANOVA with Tukey's post hoc correction for multiple comparisons compared to Control 24 hr.

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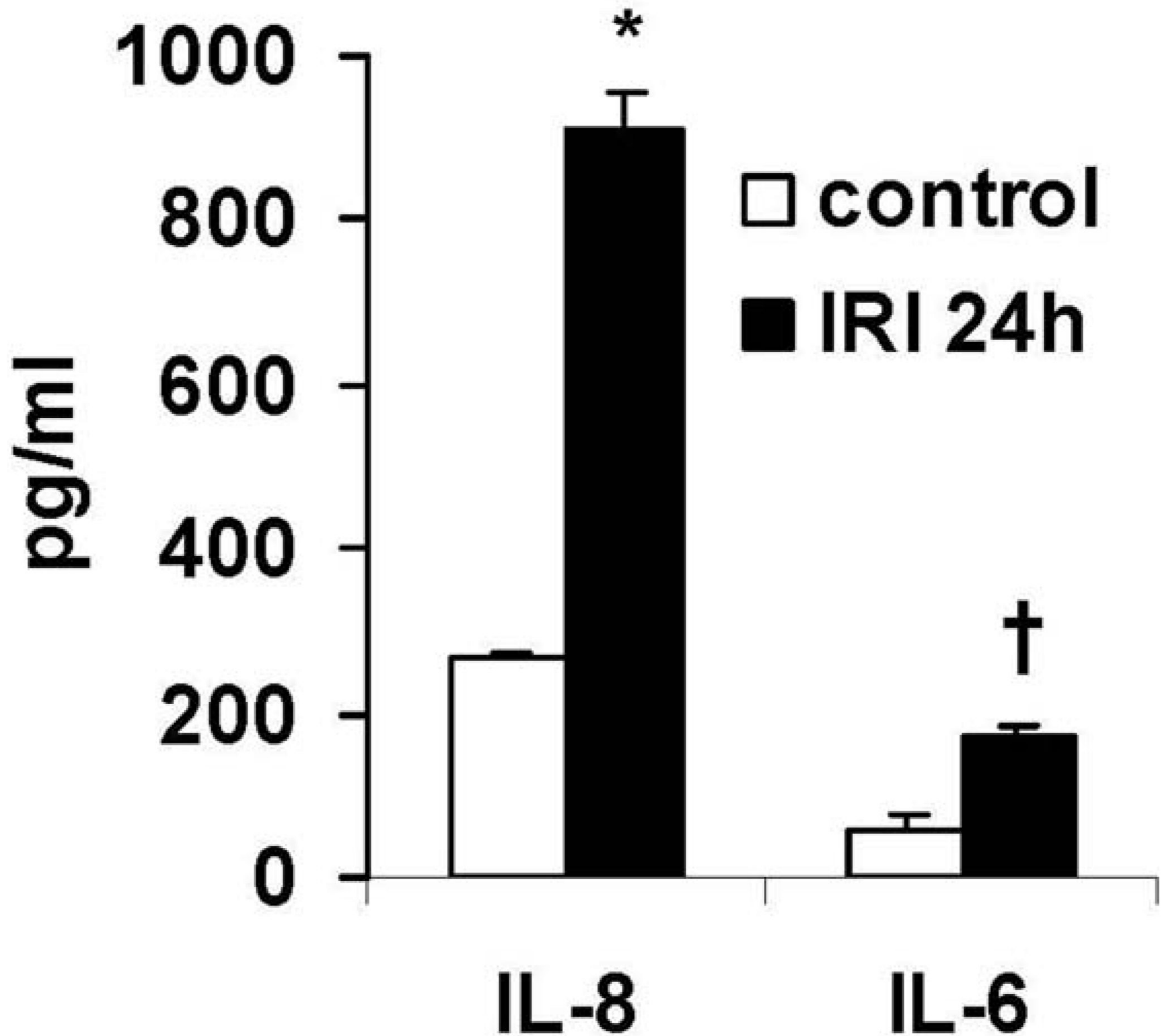

Figure 5.
IL-8 and IL-6 are both elevated in media after simulated IRI. Cytokines measured in culture media by ELISA from unstimulated HUVECs (control) and from HUVEC dishes 24 hours after the initiation of the experiment. ELISA performed in triplicate from two culture dishes (n = 6 assessments). mean ± SEM. * p<0.000005, † p<0.006 unpaired t test.

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Table 1

Photographs of groups were viewed by a cytoskeletal expert (KB), masked to group identity, who evaluated actin cytoskeleton.

Time	Comments
Control	Stress fibers going across the cells.
15 min CI	Major gaps between cells. Actin stress fibers not very prominent.
4 hr CI	Gaps between the cells are obvious. Actin is peripheral and weak.
End WI	Actin is peripheral. Some gaps are evident.
15 min rep	Actin organized around cell periphery clearly marking the cell border. Few gaps.
1 hr rep	Actin organized in prominent stress fibers, some peripheral, some crossing cells. A few gaps?
4 hr rep	Monolayer looks intact with no gaps. Actin is enriched at the borders but dispersed throughout the cells
24 hr rep	Actin stress fibers crossing the cells.