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Human Microvascular Endothelial Synthesis of Interleukin-8 During In Vitro Ischemia and Reperfusion

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Abstract These studies were undertaken to evaluate human microvascular endothelial cell (MEC) synthesis of interleukin-8 (IL-8), a potent neutrophil chemoattractant, under in vitro conditions of ischemia and reperfusion. IL-8 and other related CXC chemokines are believed to mediate tissue injury in a variety of pathologic conditions in humans. MEC grown on microcarrier beads were exposed to 3 or 6 h of in vitro ischemia followed by 2 h of reperfusion. Conditioned medium, MEC protein, and total RNA extracts were assayed for IL-8 using an ELISA. During ischemia alone, MEC increased intracellular, but not extracellular levels of IL-8 secretion. In contrast, reperfusion markedly stimulated both intracellular and extracellular IL-8 secretion. Neither 3 h of ischemia alone or followed by reperfusion altered steady-state levels of IL-8 mRNA when compared to pre-ischemic levels. In contrast, after 6 h of ischemia alone and ischemia followed by reperfusion, IL-8 mRNA was increased eight- and sixfold, respectively, when compared to pre-ischemic levels. These studies demonstrate an inverse relationship between the rate of IL-8 protein secretion and the steady-state levels of IL-8 mRNA during ischemia and reperfusion. During ischemia and reperfusion both the increase in cell-associated IL-8 protein and the release of IL-8 into the medium is dependent on de novo protein synthesis rather than the intracellular accumulation of IL-8. These experiments indicate that post-ischemic modulation of IL-8 release and synthesis following ischemia reperfusion will require strategies directed towards inhibition of IL-8 transcription and in depth knowledge of the mechanisms regulating IL-8 secretion. J. Cell. Biochem. 100: 412–420, 2007.

Key words: vascular endothelium; ischemia; cytokines; reperfusion injury

There is a growing body of evidence implicating the CXC chemokines as playing a primary role in the pathophysiology of a variety of inflammatory conditions [Schlondorff et al., 1997; Chandrasekar et al., 2001; Romagnani et al., 2004]. Because of its strategic location at the blood tissue interface, dysfunction of the vascular endothelium has also been implicated in the pathogenesis of inflammation and tissue injury following ischemia and reperfusion [Davies et al., 1998]. The microscopic appearance of tissue exposed to ischemia/reperfusion is notable for microvascular endothelial cell (MEC) edema, neutrophil adherence to endothelial surfaces and ongoing tissue ischemia due to obstruction of the microvasculature by platelets and neutrophils [Barie and Mullins, 1988; Belkin et al., 1989; Freischlag and Hanna, 1991; Blaisdell, 2002]. Interleukin (IL)-8, a CXC chemokine, activates neutrophil calcium translocation, chemotaxis, shape change, actin polymerization, degranulation, respiratory burst, and integrin-mediated adhesion of neutrophils [Hoch et al., 1996]. The pathologic adherence of neutrophils and platelets to the endothelial cell surface during reperfusion results in a paradoxical increase in tissue injury, commonly

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called the no-reflow phenomenon [Suval et al., 1987; Walden et al., 1990; Hickey et al., 1996]. Previous studies of MEC IL-8 synthesis performed on static tissue culture plates over prolonged periods of hypoxia (24–48 h), followed by reoxygenation [Karakurum et al., 1994] suggest that these cells secrete substantial amounts of IL-8 during hypoxia, prior to reoxygenation. The studies in this report were undertaken to determine the temporal and biosynthetic pattern of IL-8 expression by human MEC under conditions of acute in vitro ischemia followed by in vitro reperfusion. MEC were used for these studies because reperfusion injury appears to be a microvascular, rather than macrovascular event [Carden et al., 1990; Carden and Granger, 2000]. There is a differential distribution of IL-8 receptors (CXCR1 and CXCR2) on micro- versus macrovascular endothelium which is directly associated with functional differences on the cellular level [Salcedo et al., 2000]. To assess IL-8 synthesis under acute (3–6 h) ischemia, we utilized a novel in vitro model of ischemia/reperfusion employing MEC grown on microcarrier beads [Watkins et al., 2004]. This model allows us to simultaneously alter oxygen tension and mechanical stress in an acute and reproducible manner. Thus, the experimental apparatus utilized in this report allows an evaluation of cellular responses to sudden drops in oxygen tension along with accumulation of metabolites (stasis), which often accompany in vivo ischemia. Static tissue culture models require a slow equilibration of hypoxic gas to displace normoxic gas in an incubator, which is a scenario unlike the situation of acute organ ischemia in vivo. Likewise, the microcarrier bead device mimics in part the in vivo scenario whereupon fresh metabolites are regularly replenished during normoxia and reperfusion. The experiments are designed to evaluate MEC synthesis of IL-8 during clinically relevant periods of ischemia reperfusion. An understanding of the pattern and locale of MEC IL-8 release will allow development of strategies to inhibit/counteract inflammation related to IL-8 synthesis.

MATERIALS AND METHODS

Cell Cultures

Frozen cultures of human dermal microvascular EC (2–4 cumulative population doublings) were obtained from BioWhittaker, Inc. (Walkersville, MD). According to the manufacturer, these cells were screened for mycoplasma, HIV, hepatitis C, and had their EC identity confirmed using indirect immunofluorescence for Factor VIII, and Di-I-acetylated LDL uptake. The cells were thawed, and grown in complete medium which contained: medium 199 supplemented with 10% (v/v) defined fetal calf serum, sodium bicarbonate (2.2 gm/L) recombinant human basic fibroblast growth factor (bFGF, 5 ng/ml, Peprotech, Rocky Hill, New Jersey) penicillin (50 U/ml), streptomycin (50 mg/ml), and fungizone (5 μg/ml), and then plated on 100 mm tissue culture plates. The cells were kept in a 37°C CO_2 (5%) incubator, fed every 48 h with complete medium, and passaged at 1:4 ratios using 0.05% Trypsin/0.125% EDTA upon reaching confluence. Passage 4–6 EC were seeded (1:4–5 split ratio) onto Cytodex 3 microcarrier beads in complete medium. The microcarrier bead cultures were fed every 48 h with complete medium and kept in a 37°C 5% CO_2 incubator. At confluence (visual confirmation) the cells were fed with experimental medium for 24 h prior to the experiment. Experimental medium was identical to complete medium except as follows: M199 was supplemented with 1% defined fetal calf serum, and 10 μM tissue culture tested fatty acid free albumin (Sigma, St. Louis, MO). No phenol red or bFGF was added to experimental medium. Confluent cultures contained approximately 10^8 cells per 6.5 ml microcarrier bead volume.

Experimental Ischemia and Reperfusion

MEC grown on microcarrier beads were exposed to a basal mechanical agitation in the microcarrier flask by stirring at 20 rpm. An experimental protocol developed in our laboratory [Watkins et al., 1995, 1996] was modified as follows to study the effects of mechanical agitation and oxygen tension on human EC function: EC were allowed to stabilize during a 2 h period of normoxia (20% O_2), during which time medium was changed with fresh normoxic experimental medium every 30 min. Cells were then exposed to 3 or 6 h of ischemia (<2% O_2, stirring at 5 rpm). During ischemia, no fresh medium was provided and a slower stirring rate was used to mimic conditions comparable to stasis in vivo. At the end of the ischemic period, 70% of the medium was removed and replaced with fresh normoxic medium to begin

reperfusion. During 120 min of in vitro reperfusion (20% O_2, 20 rpm, fresh medium every 30 min) cells were stirred at 20 rpm. In selected experiments performed to determine whether IL-8 release was from cell associated or newly synthesized protein, cycloheximide (50 μM) was added to the tissue culture flask during the final 30 min of ischemia, and throughout the reperfusion intervals. To confirm the presence of normoxic or hypoxic oxygen tensions, aliquots of medium were removed from the normoxic and hypoxic reservoirs prior to the start of the experiments and from the microcarrier bead flask at the conclusion of each interval. The oxygen tension in the medium was assessed by injecting these aliquots into an AVL 995 Blood Gas analyzer (Roche-AVL Biomedical Instruments, Roswell, GA).

IL-8 ELISA Assay

The medium aspirated from the microcarrier bead flask was aliquoted into polypropylene tubes, centrifuged at 250*g* for 10 min at 4°C, snap frozen in dry ice and acetone, then stored at −80°C prior to assay. To quantitate EC production of IL-8, an ELISA (R&D Systems, Minneapolis, MN) was utilized. Standard curves for IL-8 were generated using concentrations ranging from 3.9 to 500 pg/ml. Cell-associated IL-8 levels were assayed from sedimented cells on beads subjected to cell lysis during reperfusion. A 1 ml microcarrier bead aliquot was washed with ice cold PBS. A 200 μl aliquot was removed using a positive displacement pipette and added to 800 μl of ice-cold RIPA protein extraction buffer with freshly added protease inhibitor cocktail (Sigma P8340), mixed on a vortex mixer and incubated on ice for 10 min. The sample then was sonicated for 20 min to further disrupt the cellular extract and incubated on ice for 20 min with occasional mixing on a vortex. All the tubes were centrifuged in a micro centrifuge at 13,000*g* for 10 min at 4°C. Aliquots from the supernatant were kept at −80°C until analysis.

IL-8 mRNA Elisa

Total RNA from the cells was extracted [Chomczynski and Sacchi, 1987] from 1 ml aliquots of bead sediments collected at the end of the normoxic, ischemic, and reperfusion intervals.

Using the Quantikine mRNA ELISA kit (R&D Systems) 3 μg of total RNA samples was hybridized with biotin labeled IL-8 capture oligonucleotide probe and digoxigenin labeled detection probes in a microplate. A standard curve between 0 and 500 attmol/ml was generated for these analyses. The samples were transferred to a streptavidin-coated microplate where the RNA/probe hybrid is captured. The IL-8 mRNA was detected with an anti-digoxigenin alkaline phosphatase conjugated antibody and color was developed after adding a substrate and amplifier solutions. The optical density of the samples was measured using a microplate reader and the concentration of the gene-specific mRNA was calculated against an RNA calibrator curve.

IL-8 Northern

Total RNA (15 μg) was denatured and submitted to electrophoresis on 1.2% agarose-formaldehyde gel and transferred onto nylon membrane. After the UV cross-linking the membrane was pre-hybridized at 68°C for 30 min. A high specific activity ^{32}P dCTP labeled IL-8 cDNA probe was generated using an in vitro transcription kit (New England Biolabs, Inc.) and purified using a push column (Promega). The cDNA probe was generated by reverse transcription PCR using the following primer sequences 5′-GTT GTG AGG ACA TGT GGA AGC-3′ forward and 5′-CAA CAC AGC ACT ACC AAC ACA G-3′ reverse. The ^{32}P labeled probe was added to the membrane and hybridized for 2 h at 68°C. The membrane was washed three times with 2 × SSC buffer at room temperature, and then visualized by autoradiography.

Cell Counts

Duplicate 100 μl aliquots of sedimented beads were removed for cell counts at the beginning and end of the experiments to document endothelial cell viability as previously described [Watkins et al., 1995].

Statistical Analysis

The MEC cells' first normoxic 30-min interval was treated as a stabilization period in the bench top incubator, and the IL-8 secretion during that period was not included in the statistical analysis. All data are expressed as mean ± SE. During all experimental intervals, the absolute amount of IL-8 secreted during a 30 min time period is corrected for the amount remaining from the previous period. Levels of

IL-8 mRNA after ischemia and reperfusion were compared to normoxic, pre-ischemic levels for all experiments. Cell-associated IL-8 protein levels during normoxia, ischemia, and reperfusion were normalized to per mg total protein. Results for ANOVA and paired t tests (cell counts) were computed with the computer program Instat (GraphPad, San Diego, CA).

RESULTS

MEC IL-8 Secretion

After 30 min of normoxia, the rate of IL-8 synthesis stabilized (435 ± 108 pg/10^6 cells-30 min, n = 6, Fig. 1). During 6 h of ischemia, the rate of IL-8 synthesis did not change significantly from normoxic levels until 6 h of ischemia (15.5 ± 15.0 pg/10^6 cells-30 min, n = 6, $P < 0.02$, Fig. 1). In contrast, during reperfusion after 6 h of ischemia, there was an immediate and sustained absolute (Fig. 1) and relative (Fig. 2) increase in IL-8 release for 120 min. During reperfusion after only 3 h of ischemia, IL-8 release did not increase in a sustained and significant fashion until 60 min (Fig. 2, n = 6). When compared to cells reperfused after 3 h of ischemia, cells reperfused after 6 h of ischemia produced greater levels of IL-8 protein at every interval (Fig. 2). This finding suggests that, as the duration of ischemia increases, the rate and magnitude of IL-8 secretion during reperfusion increases. In the presence of cycloheximide after 6 h of ischemia, IL-8 secretion into medium was not detectable during the final 30 min. This finding is in contrast to the low levels of IL-8 readily detected after 6 h of ischemia in the absence of cycloheximide (15.5 ± 15.0 pg/10^6 cells-30 min, n = 6, Fig. 1). Reperfusion after 6 h of ischemia in the presence of cycloheximide also decreased the rate of IL-8 secretion (Fig. 2, n = 4). These findings suggest that MEC do not secrete substantial amounts of IL-8 during prolonged ischemia, but do secrete substantial amounts during reperfusion. Finally, IL-8 secretion during ischemia and reperfusion is dependent on de novo protein synthesis.

Effects of Ischemia and Reperfusion on Cell-Associated IL-8

Cell-associated IL-8 levels were increased after 6 h of ischemia as compared to normoxic

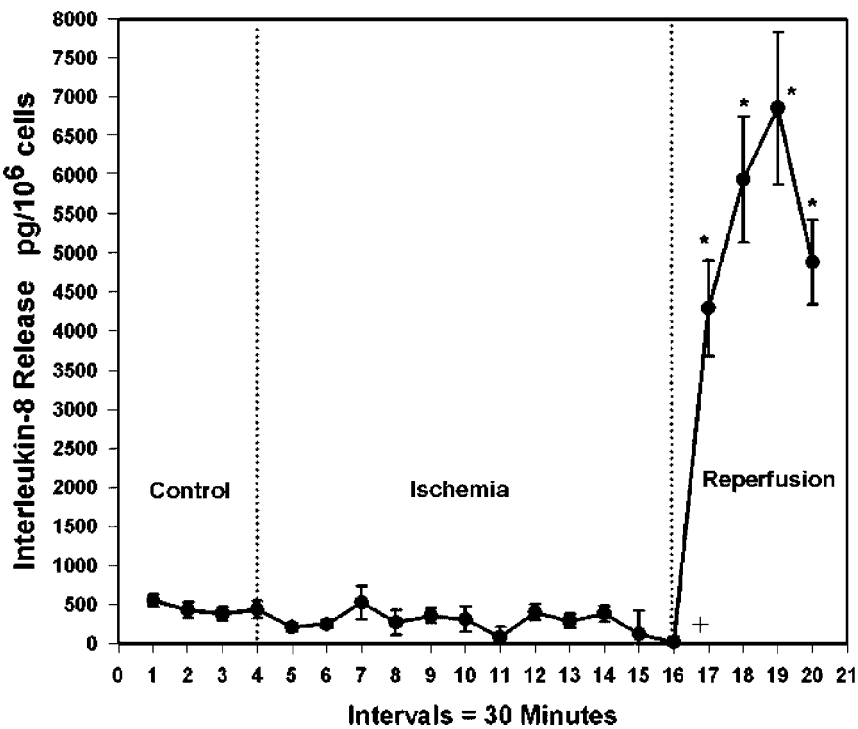

Fig. 1. Human microvascular endothelial cell secretion of IL-8 during normoxia, ischemia, and reperfusion. The rate of IL-8 secretion was stable during normoxia and ischemia. Immediately upon reperfusion, IL-8 protein synthesis was increased in a sustained and significant manner (*$P < 0.01$ vs. ischemia and normoxia, $^{+}P < 0.02$).

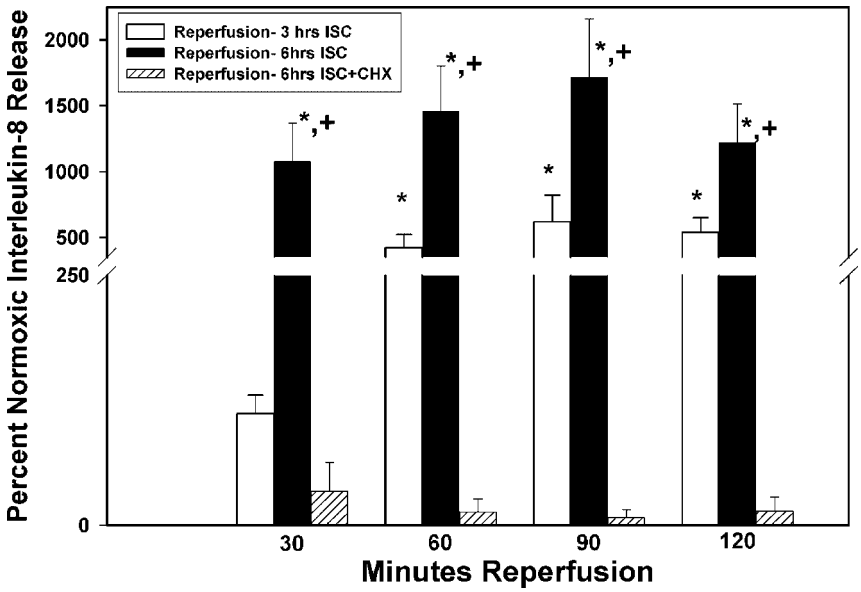

Fig. 2. Duration of ischemia modulates IL-8 secretion during reperfusion. Reperfusion after 3 h of ischemia resulted in levels of IL-8 secretion, which were not different from normoxic levels until 60 min of reperfusion (*$P < 0.01$ vs. normoxia). In contrast, after 6 h of ischemia, 30 min of reperfusion immediately increased IL-8 secretion (*$P < 0.01$ vs. control). Reperfusion after 6 h of ischemia stimulated a greater degree of IL-8 secretion than 3 h of ischemia ($^{+}P < 0.05$ vs. reperfusion after 3 h ischemia). Cycloheximide ablated IL-8 secretion during reperfusion.

conditions (normoxic 400 ± 169 vs. ischemic $1{,}545 \pm 254$ pg/mg total protein, $P < 0.01$, $n = 4$, Fig. 3). In contrast, cell-associated IL-8 levels during reperfusion were immediately increased ($5{,}116 \pm 906$ pg/mg total protein) compared to normoxic conditions (400 ± 169 pg/mg total protein, $P < 0.001$ vs. reperfusion, $n = 4$) similar to the pattern observed in conditioned medium (Fig. 3). In the presence of cycloheximide after 6 h of ischemia, cell-associated IL-8 levels were no different from levels from normoxic IL-8 levels (normoxic 400 ± 169, vs. ischemic 536 ± 102 pg/mg total protein, $P > 0.05$, $n = 4$). During reperfusion in the presence of cycloheximide, cell-associated IL-8 levels did not change substantially when compared to normoxic (no cycloheximide) or ischemic cells (with cycloheximide, Fig. 3). These experiments suggest that cell-associated IL-8 protein levels increase during ischemia, even though IL-8 secretion is markedly decreased. The increased intracellular levels of IL-8 protein during ischemia are dependent on de novo protein synthesis. During reperfusion, cell-associated levels of IL-8 increase in a pattern identical to IL-8 secretion, and is also dependent on de novo protein synthesis.

Effect of Normoxia, Ischemia, and Reperfusion on IL-8 mRNA

IL-8 mRNA levels after 2 h of normoxia alone (883 ± 256 pg/μg total RNA), 3 h ischemia alone ($1{,}813 \pm 667$ pg/μg total RNA), or 3 h of ischemia alone followed by 2 h reperfusion ($1{,}116 \pm 418$ pg/μg total RNA) were not significantly different (Fig. 4, $n = 4$). In contrast, during 6 h of ischemia IL-8 mRNA levels increased eightfold ($6{,}954 \pm 1{,}511$ vs. 801 ± 146 pg/μg total RNA, $P < 0.01$ vs. normoxia). Reperfusion after 6 h of ischemia was associated with a modest decrease in steady-state levels of IL-8 mRNA ($2{,}735 \pm 626$ pg/μg total RNA, $P < 0.001$ vs. 6 h ischemia). While steady-state levels of IL-8 mRNA during reperfusion were significantly decreased when compared to ischemia, the levels remained greater than normoxic IL-8 mRNA levels ($P < 0.05$). A representative Northern blot confirms the IL-8 mRNA ELISA analysis (Fig. 5). These experiments indicate that 3 h of ischemia followed by reperfusion does not stimulate significant increases in mRNA, despite increased levels of IL-8 protein secretion observed during reperfusion. In contrast, both 6 h of ischemia and reperfusion

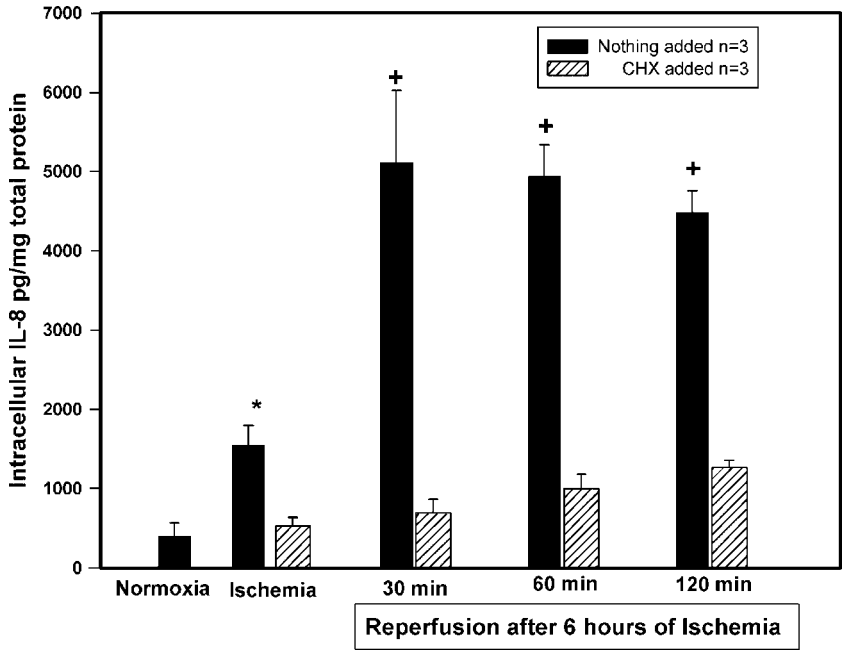

Fig. 3. Effect of reperfusion on cell-associated IL-8 levels. Cell-associated IL-8 levels were increased after 6 h of ischemia as compared to normoxic conditions (*$P < 0.01$). Reperfusion after 6 h of ischemia was associated with increased levels of cell-associated IL-8 ($^{+}P < 0.001$ vs. normoxia). Cycloheximide decreased cell-associated IL-8 levels during reperfusion, thereby implicating a role for de novo synthesis in the increase of cell-associated IL-8 during reperfusion.

result in increased steady-state levels of IL-8 mRNA.

Effect of Ischemia Reperfusion on MEC Viability

The baseline level of viable cells during normoxia was $1.1 \pm 0.2 \times 10^6$ cells/μl bead sediment. Reperfusion after either 3 or 6 h of ischemia did not decrease the number of viable cells on the microcarrier beads ($1.3 \pm 0.3 \times 10^6$ cells/μl beads 3 h ischemia reperfusion vs. $1.0 \pm 0.3 \times 10^6$ cells/μl beads, n = 6). These findings indicate that the increased levels of IL-8 detected in serum during reperfusion were not related to non-specific cell lysis.

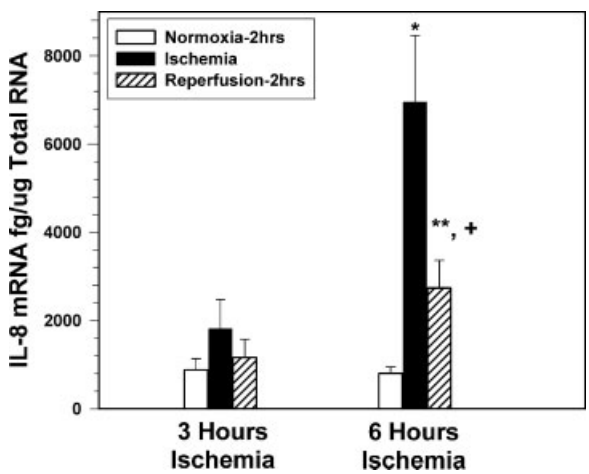

Fig. 4. Effect of ischemia reperfusion on IL-8 mRNA levels. Three hours of ischemia followed by reperfusion did not stimulate IL-8 mRNA levels. In contrast, 6 h of ischemia followed by reperfusion did markedly increase IL-8 mRNA levels (*$P < 0.01$). Reperfusion was associated with a decrease in IL-8 mRNA (**$P < 0.001$), however, the relative levels of IL-8 mRNA during reperfusion remained greater than normoxic levels ($^{+}P < 0.05$).

DISCUSSION

The data in this report shows that human MEC do not secrete substantial amounts of IL-8

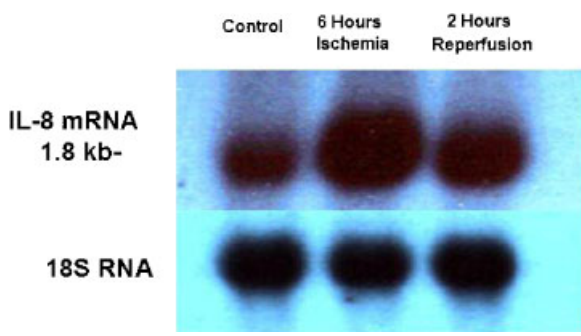

Fig. 5. Northern blot of IL-8 mRNA during normoxia, ischemia, Reperfusion. Compared to 18S mRNA levels, ischemia stimulated increased steady-state levels of IL-8 mRNA. Reperfusion decreased the levels of IL-8 mRNA.

extracellularly during ischemia. During ischemia, the intracellular levels of IL-8 increased nearly fourfold. The increase in intracellular levels of IL-8 during ischemia is based on ongoing protein synthesis, since inhibition of protein synthesis during ischemia resulted in marked decrease in intracellular levels of IL-8. These observations are important, since any attempt to modulate the effects of chemokines will require in depth knowledge of the localization of the cytokine proteins and the stimuli responsible for their secretion. The in vitro data reported herein are different from previous reports in the literature for human endothelium. In a landmark study published by Karakurum et al. [1994] human macrovascular endothelial cells were found to release substantial amounts of IL-8 by 6 h of hypoxia and throughout 4 h of reoxygenation. In contrast to the study by Karakurum et al. at no time during 6 h of in vitro ischemia was the rate of IL-8 synthesis found to be increased. The reason for the difference in the current report and the report by Karakurum et al are substantial. First, the experiments in this report employed microvascular endothelium. The vascular endothelium represents a heterogeneous cell population [Gerritsen, 1987], and ischemia-reperfusion injury appears to be primarily a microvascular event [Hickey et al., 1996; Duran et al., 1998]. In addition, since IL-8 is believed to have both angiogenic properties [Belperio et al., 2000; Li et al., 2003], and plays a pivotal role in microvascular events in the brain and lung [Matsumoto et al., 1997; Beck et al., 2001; Lee et al., 2002], the current in vitro studies were undertaken with human microvascular cells.

In addition to the differences in the site of origin (micro- vs. macrovascular), the MEC in this report were subjected to mechanical stress brought on by changing the medium and stirring of the microcarrier beads [Cherry and Papoutsakis, 1988; Cherry and Kwon, 1990]. Mechanical and shear stress alone has been shown to influence IL-8 mRNA and/or protein synthesis in endothelial cells in vitro [Kato et al., 2001; Ohki et al., 2002; Chen et al., 2003]. It is unlikely that the mechanical stress during normoxia and reperfusion alone was responsible for induction of IL-8 mRNA during ischemia or IL-8 secretion during reperfusion. The conditions of normoxia and reperfusion were identical in terms of oxygen tension and stirring rate. However, only during reperfusion after an intervening period of 6 h, but not 3 h of ischemia (where there is decreased mechanical stimulation—no stirring, no media changes) do both steady-state IL-8 mRNA levels and secretion rate for IL-8 protein increase. This implicates the duration of ischemia, rather than stirring or media changes as the primary stimulus for increased steady-state levels of IL-8 mRNA and increased rate of IL-8 secretion during reperfusion. In addition, the greatest increase in IL-8 mRNA levels was detected during ischemia, when there is no media change and decreased stirring (therefore, decreased mechanical stress).

In contrast to the pattern of increased cell-associated IL-8 and minimal secretion during 6 h of ischemia, in vitro reperfusion is associated with a marked increase in both intracellular and extracellular levels of this important chemokine. The increase in MEC IL-8 release during reperfusion is not due to a non-specific cell lysis liberating the protein from intracellular stores, since there was no measurable decrease in viable cells after 2 h reperfusion. An understanding of the pattern of human IL-8 synthesis during ischemia/reperfusion is important because in vivo human data from transplanted hearts show that coronary sinus levels of IL-8 measured following graft implantation were directly correlated with the duration of cardiac ischemia [Oz et al., 1995]. In addition to these findings in humans, canine models of myocardial ischemia also showed that myocardial injury during reperfusion was directly correlated with increased level of IL-8 mRNA [Kukielka et al., 1995]. In contrast to the in vitro findings with human MEC, in vivo myocardial ischemia in these canines was not associated with an increase in IL-8 mRNA. These differences could be related to species specificity, or a difference between the in vivo and in vitro milieu. In other studies of rodent retinas subjected to ischemia reperfusion, IL-8 mRNA and immunoreactive protein levels were increased in the vascular layers [Jo et al., 2003]. When comparing the human in vitro data with results from in vivo animal models, it is clear that the primary tendency in both settings is to increase IL-8 protein level during reperfusion, rather than ischemia. This finding means that anti-cytokine therapies geared towards interrupting the effect of IL-8 during reperfusion might be successful in ameliorating IL-8-mediated mobilization of neutrophils and other

inflammatory cells during reperfusion. It is important to note that following ischemia, and during reperfusion, there are substantial levels of intracellular IL-8. Thus attempts to modulate levels of IL-8 synthesis during ischemia and reperfusion may have to decrease IL-8 mRNA transcription and IL-8 secretion.

Since IL-8 receptors are present in vitro [Lee et al., 2002; Li et al., 2003] and in situ on vascular endothelium [Rot et al., 1996; Rot, 2003], it was possible that some IL-8 might be bound to the surface of the vascular endothelium. The fact that IL-8 mRNA levels decreased as IL-8 secretion increased suggests the existence of an autocrine feedback loop [Atta ur et al., 1999]. In addition to possessing IL-8 receptors capable of binding IL-8 protein, upon de novo synthesis, IL-8 localizes intracellularly in the Golgi [Rot et al., 1996] apparatus, from where it is secreted. In addition to this constitutive secretory pathway, a storage depot and separate regulated secretory pathway of IL-8 has been described in the Weibel–Palade bodies within endothelial cells [Wolff et al., 1998]. Weibel–Palade bodies may serve as the EC "memory" of a preceding inflammatory insult, which then enables the cells to secrete IL-8 immediately without de novo protein synthesis. Since IL-8 mRNA was increased significantly during ischemia, we suspected that it might be possible for IL-8 protein to be stored intracellularly and released non-specifically during reperfusion [Utgaard et al., 1998]. Thus, to determine whether IL-8 release during reperfusion in the medium was related to cell associated (intracellular or bound IL-8), reperfusion was performed in the presence of cycloheximide. Cycloheximide is a non-specific inhibitor of protein synthesis. We hypothesized that if the majority of IL-8 released during reperfusion originated from cell-associated stores, substantial levels of IL-8 should be observed in the medium despite the presence of cycloheximide. As shown in Figure 2, cycloheximide administered during the last 30 min of ischemia and throughout reperfusion decreased nearly all-measurable IL-8 protein in conditioned medium. Similarly, cell-associated IL-8 levels during reperfusion in the presence of cycloheximide did not increase (Fig. 4). These results implicate de novo synthesis for the increase in secreted and cell-associated IL-8 during reperfusion of human MEC.

In conclusion, these data demonstrate that that prolonged (greater than 3 h) periods of in vitro ischemia increase steady-state levels of IL-8 mRNA but do not increase the rate of IL-8 synthesis from MEC. Furthermore, during in vitro reperfusion, de novo synthesis of IL-8 protein, rather than stored or bound IL-8 is responsible for the observed increase in IL-8 protein in conditioned medium. These finding suggest that intracellular and extracellular IL-8 might be targeted during reperfusion to decrease inflammation. Since IL-8 has both proinflammatory and angiogenic properties, close attention to the consequences of in vivo blockade will be required [Belperio et al., 2000; Li et al., 2003].

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