Microvascular transport is associated with TNF plasma levels and protein synthesis in postischemic muscle

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Takenaka, Hiroaki, Hidemi Oshiro, David D. Kim, Peter N. Thompson, Atsushi Seyama, Robert W Hobson II, and Walter N. Durán. Microvascular transport is associated with TNF plasma levels and protein synthesis in postischemic muscle. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1914–H1919, 1998.—To better understand the mechanisms of ischemia-reperfusion (I/R) injury, we tested the hypothesis that protein synthesis is involved in the production of tumor necrosis factor (TNF) and in the microvascular transport changes in I/R. To evaluate the hypothesis, we inhibited protein synthesis with topically applied actinomycin D (AMD), measured I/R-induced changes in microvascular transport, and bioassayed the venous plasma levels of TNF. The rat cremaster muscle I/R model consisted of 4 h of ischemia followed by 2 h of reperfusion. Changes in transport were determined by integrated optical intensity (IOI) using FITC-Dextran 150 as tracer. Animals were separated into four groups: 1) control (C), 2) control treated with AMD (C + AMD), 3) I/R, and 4) I/R treated with AMD (I/R + AMD). The mean (±SE) maximal IOI in C and C + AMD were 3.0 ± 1.0 and 3.7 ± 0.7 units, respectively. I/R elevated mean maximal IOI to 21.8 ± 1.9 units (P < 0.05 vs. C, C + AMD, I/R + AMD). Treatment with AMD reduced the I/R-induced mean maximal IOI to 9.7 ± 2.0 units (P < 0.05 vs. I/R). In I/R group, plasma TNF levels increased (relative to preischemia baseline) immediately after the release of the vascular occlusion to 250 pg/ml and reached a peak value of 342 pg/ml at 60 min of reperfusion. In the I/R + AMD group, AMD reduced TNF increase to 44 pg/ml. The C and C + AMD groups showed no differences in TNF values during the 6 h of observation. We conclude that protein synthesis and TNF generation are at least partially involved in I/R-induced changes in microvascular transport.

ischemia-reperfusion injury; actinomycin D; deoxyribonucleic acid transcription; skeletal muscle; tumor necrosis factor

SKELETAL MUSCLE is unique in its ability to tolerate long periods of ischemia compared with other organs such as brain, kidney, or heart. However, acute interruption of arterial blood flow to the extremities in humans is associated with high morbidity, frequent limb loss, and sometimes death. Reduction or interruption of arterial blood flow to skeletal muscle leads to a deficit in nutrient supply to cells, decreased energy production, and ultimately cellular dysfunction and death. The extent of the injury is not limited to the ischemic time. Reestablishment of blood flow, which is essential for maintenance of cell life, carries with it an exaggeration of these phenomena, resulting in further injury to the tissue and dissemination of the accumulated products to the body.

Various factors have been suggested as causes of tissue damage in ischemia and reperfusion (I/R) of skeletal muscle, but the cellular mechanisms of action have not been fully elucidated. In particular, there is suggestive evidence that tumor necrosis factor (TNF) may play a significant role in these cellular interactions. TNF may be produced by postischemic tissue inasmuch as the venous effluent from postischemic rat hindlimb perfused with a nonrecirculating crystalloid-based buffer contains a high level of TNF. The tissue sources for TNF may contribute to a reverberant cycle in which neutrophil activation by TNF causes the upregulation of cell adhesion molecules on both neutrophils and endothelial cells, then adherent neutrophils migrate to the interstitium and generate oxygen-derived free radicals and release toxic enzymes. Activated leukocytes further generate cytokines such as TNF and interleukin-1.

Thus this circuit will result in exaggerated microvascular insults. Despite this conceptual framework, the correlations between cytokine levels and microcirculatory dysfunction have not been investigated systematically in striated muscle.

Because TNF is present in venous effluent of postischemic muscle, we reasoned that probably new synthesis of TNF occurred during ischemia. If I/R damage involves protein synthesis, then treatment with agents that interfere with protein synthesis may attenuate the reperfusion injury of postischemic tissue. In this study, we tested the hypothesis that newly synthesized proteins participate in and promote microcirculatory dysfunction in rat skeletal muscle I/R injury. We employed actinomycin D, an antibiotic that binds to DNA and blocks the movement of RNA polymerase and thus prevents transcription, to prevent synthesis of new proteins during I/R phases in rat cremaster muscle. We posed the following questions: 1) Does protein synthesis occur in I/R? 2) If so, does it influence the production of TNF? 3) Are protein synthesis and TNF levels associated with changes in microvascular transport? We hypothesized that 1) TNF levels will be increased in I/R, 2) inhibition of protein synthesis should block the increase in TNF, and 3) inhibition of protein synthesis, and of the elevated TNF levels, should diminish the impact of I/R on microvascular transport.

To test the above hypotheses, the objectives of this study were 1) to inhibit protein synthesis during the ischemic and reperfusion periods and 2) to evaluate the effects of protein synthesis inhibition on TNF levels and on microvascular transport. Our results support the concept that protein synthesis and TNF levels are associated and promote changes in microvascular transport in the rat cremaster muscle subjected to I/R.
MATERIALS AND METHODS

I/R model. This study was approved by the Animal Care and Use Committee at the New Jersey Medical School and complied with National Institutes of Health “Guide for the Care and Use of Laboratory Animals” [Department of Health and Human Services Publication No. (NIH) 85–23, Revised 1985]. Twenty-eight male Wistar Furth rats, weighing 140–200 g, were anesthetized with pentobarbital sodium (60 mg/kg ip). Body temperature was maintained at 37°C by placing the animal on a heating pad. Trapeotomy was performed to facilitate ventilation. The right jugular vein was cannulated with a catheter (PE-50; Clay Adams, Parsippany, NJ) for administration of fluorochrome tracer and supplementary doses of anesthetic. The right femoral vein was cannulated with a catheter (PE-10), and the catheter was advanced to collect blood samples from the inferior vena cava.

The left cremaster muscle was prepared for fluorescent intravitral microscopy as previously described (6, 24, 25). In brief, the muscle was approached through a scrotal incision, separated from the skin, and cleared of connective tissue. The cremaster was incised longitudinally. Vessels connecting the cremaster and epididymis were divided with thermocautery. Briefly, the muscle was approached through a scrotal incision, separated from the skin, and cleared of connective tissue. The testis was placed in the abdominal cavity. The cremaster and epididymis were divided with thermocautery.

With the use of Evans blue as a visual index, no visible stagnant areas were present in either chamber. In addition, vascular reactivity of the cremaster muscle preparation was normal.

Microscopy. Observations were made with a Nikon microscope equipped with a television camera (Optronics, TEC-470) suitable for both trans- and epi-illumination. The fluorescence epi-illumination system consists of a 100-W mercury direct-current lamp source, a fluorescein exciter filter, a dichroic mirror, and a barrier filter. The optics include ×6.3 and ×32 long-distance working objectives and ×10 oculars.

Measurement of microvascular transport. Microvascular transport of macromolecules was determined by intravital fluorometry using computer-assisted digital processing (2, 3). Three to five selected areas in the rat cremaster muscle were videorecorded during the experiment. Each experimental frame was played back and digitized into 512 by 512 picture elements (pixels) with an Image 1 image digitizer. Each pixel was associated with an eight-bit gray scale (a number between 0 and 255, in which 0 is black and 255 is white). Integrated optical intensity (IOI) was calculated as a measure of the total gray scale value in a selected area and averaged in 180 by 150 pixels. The interstitial IOI of the fluorescent tracer reflects its permeation through the microvascular wall (2, 3).

TNF assay. Plasma TNF activity was measured by biosay (19, 22). Briefly, 1.5 × 10⁶ WEHI 164 cells (ATCC, Rockville, MD) were added to each well of a 96-well microtiter plate (Costar, Cambridge, MA) with 100 µl of the diluting media and 1 µg/ml AMD. The diluting media consisted of RPMI 1640 (Sigma Chemical), 1% fetal bovine serum (GIBCO, Grand Island, NY), 2 mM L-glutamine (Sigma Chemical), 50 µg/ml gentamicin (GIBCO), 10 mM HEPES (GIBCO), 1 mM sodium pyruvate (Sigma Chemical), and 2 × 10⁻² M mercaptoethanol (Sigma Chemical). Plates were incubated for 4 h in a humidified 5% CO₂ atmosphere at 37°C. Recombinant murine TNF-α aliquots (25 µl, 50 ng/ml; Boehringer Mannheim, Indianapolis, IN) were triplicated with 12 serial dilutions to generate a standard curve. Experimental samples (25 µl) were triplicated with eight serial dilutions. The plates were incubated for 24 h in the same conditions. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical) was then prepared at 5 mg/ml, and 25 µl were added to each well. After 2 h of incubation at the same
conditions, 100 µl of lysing buffer (10% SDS, 50% dimethylformamide, pH 4.7, Sigma Chemical) were added to each well to stop the color change. The plates were placed in the dark at room temperature for longer than 8 h. The plates were then read at 570 nm on a Dynatech 5000 microplate reader. This bioassay measures both TNF-α and TNF-β. The TNF activity of the experimental samples was calculated from a standard curve generated using TNF-α as a calibration index.

Experimental groups. Four experimental groups were studied. The first group was a sham group used to evaluate the effects of time and surgery on the preparation and was not subjected to ischemia and reperfusion (control, C; n = 7). The second group was used to assess the effect of AMD on the sham preparation (C + AMD; n = 7). The third group was subjected to I/R to determine the extent of microvascular dysfunction in the muscle, using changes in IOI as an index (I/R; n = 7). The fourth group was subjected to I/R and treatment with AMD to examine the effects of AMD on microvascular dysfunction (I/R + AMD; n = 7).

Experimental protocol. The protocol used in this study is shown in Fig. 2. After 45 min of stabilization, three to five postcapillary venules were randomly selected and videorecorded every 10 min during the baseline period. A blood sample (0.2 ml) was drawn before the induction of ischemia. After the basal data collection period, the cremasteric vascular pedicle was cross-clamped to interrupt blood flow to the muscle for global ischemia in the third and fourth groups of animals. Ischemia was maintained for 4 h to allow for potential induction of protein synthesis-related processes. After ischemia, blood flow was restored by releasing the vascular pedicle clamp. Blood samples were collected just after restoration of blood flow (time 0) and at 60 and 120 min after reperfusion. The selected areas in the cremaster muscle were videorecorded every 10 min throughout 2 h of reperfusion. In group C + AMD and group I/R + AMD, the muscle was superfused with a buffer containing 10^{-6} M AMD during both ischemia and reperfusion periods. In both control groups C and C + AMD, the cremaster muscle was subjected to superfusion with bicarbonate buffer for 6 h after the stabilization period to time match the 4 h of ischemia plus 2 h of reperfusion in the I/R groups.

Statistical analysis. All data are expressed as means ± SE. Comparisons between groups were performed with the Kruskal-Wallis one-way ANOVA. Each group mean value was subjected to the Student-Newman-Keuls post hoc test. Statistical significance for the differences was set at P < 0.05.

RESULTS

Quantitative changes in microvascular transport (IOI). The statistical analysis of the changes in microvascular transport is displayed in Fig. 3. The data represent the analysis of the reperfusion period. The time-matched controls (without and with AMD) reflect the measurement of IOI in the last 2 h of a 6-h sham muscle preparations. The maximal mean IOI values in the C and C + AMD control groups were 3.0 ± 1.0 and 3.7 ± 0.7 units, respectively. There was no significant difference between two groups. I/R elevated mean maximal IOI to 21.8 ± 1.9 (P < 0.05 vs. C). Treatment with AMD (I/R + AMD group) inhibited the I/R-induced increase in IOI to a mean maximal value of 9.7 ± 2.0 but did not abolish the change in microvascular transport (P < 0.05 vs. I/R and vs. C).

Fig. 2. Experimental protocol. Diagram shows time sequence of events in this model of ischemia-reperfusion (I/R). AMD, actinomycin D.

Fig. 3. Statistical analysis of impact of inhibition of protein synthesis on microvascular transport. Actinomycin D (AMD) did not change baseline microvascular transport (control + AMD). Integrated optical intensity (IOI) values for control groups represent final 120 min of a 6-h sham experimental preparation. Influence of ischemia-reperfusion (I/R) on transport of macromolecules is demonstrated by I/R group. Topical superfusion with 10^{-6} M AMD attenuated significantly increase in postischemic microvascular transport (I/R + AMD). *P < 0.05, ***P < 0.001 compared with control group. """"P < 0.001 compared with I/R + AMD group.
Plasma TNF levels. The time course of plasma TNF is displayed in Fig. 4. The plasma levels of TNF remained constant throughout the final 120-min period in the C and C + AMD control groups. This 120-min period is equivalent to the reperfusion period in the I/R groups. There was no significant difference in plasma TNF levels between the two control groups. I/R significantly elevated TNF plasma levels. In the I/R group, the plasma TNF levels increased immediately after the release of the vascular clamp (time 0) to 250 pg/ml and reached a peak value of 342 pg/ml at 60 min after reperfusion. This value declined but remained still significantly elevated (259 pg/ml) at 120 min of reperfusion. Treatment with AMD inhibited the changes in plasma TNF levels. In the I/R + AMD group, a slight increase of TNF (44 pg/ml) was observed at reperfusion and subsequently decreased with time and became similar to the pres ischemic mean value.

DISCUSSION

Our main conclusions are as follows: 1) protein synthesis is associated, at least partially, with microvascular transport changes in postischemic striated muscle; and 2) TNF may participate in the events associated with changes in microvascular transport in I/R. These conclusions are supported by the reduction of the increment of interstitial IOI and the inhibition of the changes in TNF plasma levels after treatment with AMD in our I/R model.

TNF plasma levels. We observed that plasma TNF levels increased immediately after the onset of reperfusion and remained elevated throughout the experimental period. Sternbergh et al. (23) showed similar results in isolated rat hindlimbs that were perfused with a noncircuiting crystalloid-based buffer. They showed that reperfusion of the rat skeletal muscle resulted in a very rapid and short-lived increase of plasma TNF bioactivity. The exact mechanisms of the increase in plasma TNF remain unknown. Endothelial cells and tissue macrophages are possible sources of the trigger cytokines that initiate the interaction between the endothelial cells and leukocytes. The biosynthesis of TNF is tightly regulated by transcriptional and translational mechanisms (8). TNF does not exist in a stored form in macrophages (8); however, production of active TNF occurs in a period as short as 15 min after exposure to an inflammatory stimulus (23). In the context of our experiments, TNF levels were elevated in the plasma sample taken at time 0, i.e., immediately at the onset of reperfusion. Therefore, we speculate that the rapid increase of plasma TNF level observed in the initial phase of reperfusion is most likely due to washout of TNF generated during ischemia. An alternative explanation would be to invoke a role for mast cells in I/R. Mast cells can release both preformed and newly synthesized TNF-α upon activation of the cells (14, 15).

In our model, TNF levels showed a peak value at 1 h of reperfusion and remained significantly elevated for 2 h of reperfusion. Seekamp et al. (22) reported that jugular vein blood samples showed a significant plasma TNF increase at ~1 h of reperfusion in the rat hindlimb ischemia model. They also demonstrated that TNF levels reached their peak at 2 h of reperfusion and were still significantly elevated after 4 h of reperfusion. In our model, in which ischemia is effected in a tissue with a mass of ~200 mg, local TNF production may not be enough to sustain the high TNF levels measured during the reperfusion phase. We speculate that locally produced TNF is carried to the systemic circulation and may promote leukocyte and macrophage activation in remote organs such as lung and liver. Alveolar macrophages and liver Kupffer cells may be activated and participate in the systemic production of TNF. Systemically generated TNF may account for the sustained elevation of plasma TNF during the reperfusion period.

Microvascular transport, TNF, and protein synthesis. IOI values increased gradually with time during the reperfusion period and reached their maximal value at 120 min after reperfusion. Treatment with AMD significantly decreased but did not abolish the extravasation of FITC-Dx 150 induced by I/R. This observation is of interest because the TNF data indicate the dose of AMD was sufficiently efficacious to block protein synthesis. Thus our data support a partial role for protein synthesis in the changes in microvascular transport induced by I/R.

We have established an association between TNF synthesis and changes in microvascular transport. Our experiments demonstrate that 1) as TNF plasma levels are elevated by I/R, the blood-tissue transport of macromolecules is increased; and 2) upon inhibition of TNF production during I/R, the rate of macromolecular transport is reduced. On the basis of our data and reports by others, it is reasonable to conclude that ischemia stimulates elevated levels of TNF, which in turn activates the endothelium directly and indirectly by enhancing leukocyte adhesion. The sum of direct activation and leukocyte adhesion and migration would lead to increased microvascular transport. However,
more direct experiments are necessary to probe and demonstrate this potential cause-effect relationship. Because AMD reduced but did not abolish the I/R-induced increase in IOI, our data also support the involvement of additional factors that do not require de novo protein synthesis. This conclusion agrees with data from several laboratories. We and others have shown that the vascular damage associated with I/R can be significantly ameliorated by several pharmacological interventions designed to block prostanoid production (7), to inhibit formation of free radicals (16), and to reduce leukocyte count (4, 19, 26). An additional attractive candidate for the role of promoter of transport changes is platelet-activating factor (PAF). Synthesis of PAF does not require DNA transcription because PAF is rapidly synthesized from membrane phospholipid by the activity of phospholipase A2 and acetyltransferase.

We speculate that oxygen free radical released from endothelial cells and polymorphonuclear neutrophils early in the reperfusion phase may activate the production of PAF. This phospholipid is a potent chemotactic agent and promotes adhesion between endothelial cells and leukocytes (10) and directly increases microvascular transport in a dose-response manner (9, 10). Upon PAF stimulation, neutrophils release lysosomal enzymes and superoxide anions and generate arachidonic acid metabolites. Additionally, PAF enhances the production of TNF by monocytes and macrophages (5, 11). This chain of events amplifies the PAF effects. Our speculation is supported by the experimental observations that blockade of PAF receptors decreases adhesion of leukocytes (12) and changes in macromolecular transport (20) in I/R.

In summary, our results demonstrate that inhibition of protein synthesis eliminates the I/R-increased levels of TNF and significantly diminishes the changes in microvascular transport induced by ischemia and reperfusion in striated muscle.

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