TREATMENTS FOR MYOCARDIAL ischemia are directed toward the restoration of blood flow and oxygen delivery, i.e., reperfusion. However, reperfusion of ischemic tissue is typically accompanied by an oxidative burst of reactive oxygen species (ROS) production (2) that counteracts the restorative effects of reoxygenation. Indeed, ischemia-reperfusion (I/R) injury likely plays a major role in many pathophysiologicals, including shock, coronary artery bypass surgery, and solid organ transplantation, and is a major factor in the pathogenesis of life-threatening diseases (2). Reperfusion injury affects recovery of patients following coronary bypass surgery and cardiopulmonary bypass. Specifically, cardiac surgery involving cardiopulmonary bypass and I/R is associated with significant mortality and morbidity, despite recent advances in technology and technique (19, 32).

Inflammation is the first phase in the postischemic period in skeletal muscle (9, 10). Duran et al. introduced the strategy of applying molecular biology agents (monoclonal antibodies) at the time of reperfusion (9, 10). It argues that this strategy applies to all patients and not exclusively to elective surgery (as is the case for application of agents before inducing ischemia). Importantly, these studies show the relevance of leukocyte “invasion” in postischemic tissue damage (11). Tumor necrosis factor-α (TNF-α) is an important factor in I/R, which is synthesized during the period of ischemia (26–28). Previously, our laboratory observed (32) a role for TNF-α in myocardial I/R-induced endothelial injury using a protocol in which antibody neutralization of the cytokine was achieved before I/R. Such a protocol, however, does not provide answers regarding a potential therapeutic effect of neutralizing TNF-α, which should be accomplished at the time of reperfusion. Because TNF-α plays a direct role in the adhesion of circulating inflammatory cells (19), and inflammatory cells are thought to play a role in I/R injury, we hypothesized that neutralization of TNF-α at the time of reperfusion exerts a protective effect on I/R injury by limiting the invasion of inflammatory cells.
METHODS

Marine I/R model. The procedures followed were approved by and in accordance with approved guidelines set by the Laboratory Animal Care Committee at University of Missouri. This study utilized 12- to 16-wk-old, 25- to 35-g wild type (WT) control mice of either sex. The strain of WT control mice is B6, 129SvF2J (stock no. is 101045, Jackson Laboratory, Bar Harbor, ME). The surgical protocol was performed similar to methods described previously (13, 31, 32) with minor modification. After 30 min of the left anterior descending coronary artery occlusion and 90 min of reperfusion, the heart was harvested and used for functional study or other assays. A sham group was subjected to the same surgical interventions without performing occlusions. All mice were maintained on a 12:12-h light-dark cycle with food and water ad libitum.

Induction of neutropenia. WT mice were rendered neutropenic (20) by an intraperitoneal injection of a 1:20 dilution of antineutrophil antiserum. Mice were bled before and 24 h after the injection, and blood neutrophil counts were performed to confirm neutropenia. Briefly, 5 μl of blood were collected from the tail vein, blood cells were diluted and stained in Kimura stain, and neutrophils were counted with the aid of a hemocytometer. Pilot experiments revealed that mice remained neutropenic for ~45 h after the administration of the antiserum.

mRNA expression of TNF-α by real-time PCR. Total RNA was extracted from coronary arterioles using Trizol reagent (Invitrogen), and 1.0 μg total RNA was processed directly to cDNA synthesis using the SuperScript III Reverse Transcriptase (Invitrogen). cDNA was amplified with the use of qRT-PCR Kit with SYBR Green (Invitrogen). The primer set for specific amplification of TNF-α (13, 32) was designed in previous studies. Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. The housekeeping gene β-actin was used for internal normalization. The mean threshold cycle (CT) values for both the target (TNF-α) and internal control (β-actin) genes were determined. Data were calculated by 2-ΔΔCT method (ΔΔCT = CT_TNF-α – CT_β-actin) (23). Results were presented as fold change of transcripts for TNF-α in I/R mice normalized to internal control (β-actin), compared with sham mice (defined as 1.0-fold). Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Western blotting. For Western blot analysis, isolated coronary arterioles (4–6 vessels per sample) from sham, I/R, and I/R mice treated with anti-TNF-α at the time of reperfusion were homogenized and sonicated in lysis buffer (Cellytic MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed with use of BCA Protein Assay Kit (Pierce), and equal amounts of protein (4 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). TNF-α and nitrotyrosine (N-Tyr) protein expression were detected by Western blot analysis with use of TNF-α primary antibodies (1:200, 4° overnight; Santa Cruz) or N-Tyr (an indicator for peroxynitrite-mediated tissue injury) primary antibodies (1:500, 4° overnight; Abcam). Horseradish peroxidase-conjugated goat anti-mouse was the secondary antibody (1:1,000; Abcam). Signals were visualized by enhanced chemiluminescence (Amersham) and quantified by Quantity One (BioRad Ver- sadoc imaging system).

Immunohistochemical analysis. Immunohistochemistry was used to identify and localize proteins in sections of arteries or myocardial tissue. Fresh hearts were embedded in optimum cutting temperature compound and were cut into 5 μm sections. Frozen sections were fixed in ice-cold acetone for 10 min and were incubated with blocking solution (10% donkey serum in 1% BSA), then incubated with TNF-α (goat polyclonal to TNF-α, R&D), and endothelial cell marker, von Willebrand factor (vWF) (rabbit polyclonal to vWF, Abcam), or smooth muscle α-actin (rabbit polyclonal to α-smooth muscle actin, Abcam), or anti-N-Tyr (Abcam) primary antibodies, then incubated with a secondary fluorescent antibody (donkey polyclonal to goat Texas red and donkey polyclonal to rabbit FITC, Abcam). Sections were finally mounted in an anti-fading agent (Slowfade gold with 4,6-diamidino-2-phenylindole, Invitrogen). Slides were observed and analyzed using a fluorescence microscope with a ×40 objective (Olympus IX81, see Fig. 2) or using a fluorescence microscope with a ×63 objective (Leica microscope, see Fig. 6). For every section, a negative control (without primary antibody) was performed.

Functional assessment of isolated coronary arterioles. The techniques for identification and isolation of coronary microvessels were described in detail previously (13, 31). Briefly, after I/R, the heart was excised and immediately placed in cold (4°C) saline solution. Each coronary arteriole (40–100 μm in internal diameter) was isolated and cannulated with glass micropipettes, pressurized to 60-cmH2O intraluminal pressure, and bathed in physiological salt solution (PSS). The inner diameters of coronary arterioles were measured using video microscopic techniques. After a stable baseline tone was developed (i.e., spontaneous constriction to 60–70% of maximal diameter), the experimental interventions were performed. The concentration–diameter relationships for acetylcholine (ACh, 1–10 μmol/l) and sodium nitroprusside (SNP, 1 μmol/l to 1 μmol/l) were established.

Two protocols were then performed to assess the involvement of TNF-α in the vasodilations before reperfusion. First, the contribution of TNF-α in these vasodilations was examined by administration of neutralizing antibodies to TNF-α (anti-TNF-α) before reperfusion for 90 min (1.6 mg/mouse iv). Anti-TNF-α used in these studies was IgG goat polyclonal antibody prepared to be essentially lipopolysaccharide free. Second, to address the roles of superoxide (O2−) before reperfusion in I/R-induced vascular dysfunction, the nitric oxide-mediated response of coronary arterioles was assessed in the absence and presence of xanthine oxidase inhibitor allopurinol (15 mg/kg iv) or/and NAD(P)H oxidase inhibitor apocynin (25 mg/kg iv) immediately before initiating reperfusion for 90 min. All drugs were administered before reperfusion (intravenously) in these functional studies.

MPO activity. Mouse hearts were removed to determine myocardial MPO activity using mouse MPO ELISA kit (Cell Sciences). This kit is a solid-phase, enzyme-linked immunosorbent assay based on the sandwich principle. Samples and standards were incubated in microtiter wells coated with antibodies recognizing mouse MPO. Color develops proportionally to the amount of MPO present in the sample. The enzyme reaction was stopped by the addition of citric acid, and the absorbance at 450 nm was measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance vs. the corresponding concentrations of the standards. The MPO concentration of samples with unknown concentrations, which was run concurrently with the standards, is then determined from the standard curve.

Measurement of O2− by electron paramagnetic resonance spectroscopy. The homogenate (4–6 isolated coronary arterioles) was prepared as described previously (13, 31). Superoxide quantification from the electron paramagnetic resonance (EPR) spectra was determined by double integration of the peaks, with reference to a standard curve generated from horseradish peroxidase generation of the anion from standard solutions of hydrogen peroxide, using p-acetamidophenol as the co-substrate, and then normalized by protein concentration. Twenty-four hours after the administration of the antineutrophil antibody, O2− was measured in neutrophic I/R mice. NAD(P)H oxidase inhibitor MFH244 was administered as a slow bolus (30 mg/kg ip) 10 min before reperfusion in WT I/R mice for measuring of O2−.

Chemicals. All drugs were obtained from Sigma, except as specifically stated. Anti-neutrophil antiserum was obtained from Inter-Cell Technologies (Hopewell, NJ). MFH244 (new designator; original designated “VF244”) was a gift received from Dr. Howard Elford (Molecules For Health, Richmond, VA). ACh, SNP, allopurinol, and apocynin were dissolved in PSS for functional studies and in phosphate-buffered saline for fluorescence detection. Allopurinol was dissolved in water. These drugs were then diluted in PSS to obtain the desired final concentration. Vehicle control studies indicated that...
these final concentrations of solvent had no effect on the arteriolar function.

Data analysis. At the end of each experiment, the vessel was relaxed with 100 μmol/l SNP to obtain its maximal diameter at 60-cmH²O intraluminal pressure (13, 31). All diameter changes in response to agonists were normalized to the vasodilation in response to 100 μmol/l SNP and expressed as a percentage of maximal dilation. All data are presented as means ± SE, except as specifically stated (e.g., as mean ± SD for molecular study). Statistical comparisons of vasomotor responses under various treatments were performed with one-way or two-way ANOVA, and intergroup differences were tested with Bonferroni inequality. Significance was accepted at P < 0.05.

RESULTS

Ischemia increased TNF-α mRNA and protein expression in murine coronary arterioles. The mRNA (Fig. 1A) and protein expression (Fig. 1B) of TNF-α in left ventricular coronary arterioles of sham, I/R, and I/R groups treated with anti-TNF-α at the time of reperfusion in WT mice significantly increased in the I/R group.

Cellular source of TNF-α expression in I/R injury. We used a double immunostaining of TNF-α and a vascular smooth muscle cell marker α-actin or endothelial cell marker vWF to explore if TNF-α was localized in vascular wall in I/R. As shown in Fig. 2, D–F, the TNF-α and α-actin were almost completely colocalized in I/R, indicating that the TNF-α was expressed by vascular smooth muscle cells following I/R injury. TNF-α and vWF (Fig. 2, G–I) were not colocalized in I/R, indicating that the TNF-α was not expressed in endothelial cells following I/R injury.

Second, we performed immunohistochemistry for TNF-α and MPO (expressed by neutrophil cells) to determine whether TNF-α was colocalized with MPO in coronary microvessels in I/R injury. Our data show that TNF-α in I/R was not colocalized with MPO (data not shown), indicating TNF-α may not be expressed in neutrophil cells in I/R injury.

We also performed immunohistochemistry for TNF-α and macrophages, or TNF-α and mast cells, to determine whether TNF-α was produced by inflammatory cells (macrophages or mast cells) in I/R injury. Our results show that there were no signals for macrophages (data not shown) in sham animals, but there were signals in mast cells in sham animals (data not shown). Our results (WT-I/R) also show that the increased staining of TNF-α was colocalized with the macrophages (data not shown) and mast cells (data not shown).

Our results from the negative control experiment show an absence of staining in coronary vessels using only the secondary antibodies (Fig. 2, K–M); these results demonstrate the specificity of our immunostaining.

Role of TNF-α in I/R-induced vascular dysfunction. Our laboratory previously showed that function of endothelial cells was impaired, but function of smooth muscle cells was preserved in I/R (13, 32). Anti-TNF-α (1.6 mg/mouse iv, administered before initiating reperfusion) partially restored Ach-induced coronary arteriolar dilation following I/R (Fig. 3A), but anti-TNF-α did not affect vasodilation in the sham group.

I/R-induced O₂⁻⁻ production in murine coronary arterioles. Administration of xanthine oxidase inhibitor allopurinol or NAD(P)H oxidase inhibitor apocynin before reperfusion partially restored vasodilation to Ach in I/R. Furthermore, allopurinol or apocynin did not affect Ach-induced vasodilation in sham groups (Fig. 3B). However, the simultaneous application of allopurinol and apocynin did afford more “protection” than that seen with either agent alone. Interestingly, endothelial function during I/R was not significantly affected in neutropenic animals, or by the neutrophil NAD(P)H oxidase inhibitor MFH244 (Fig. 3C).

EPR spectroscopy shows (Fig. 4) O₂⁻⁻ production increased following I/R compared with the sham group, and administration of anti-TNF-α or allopurinol and apocynin before reperfusion reduced the expression of O₂⁻⁻ in I/R mice to the level observed in the sham group. Importantly, O₂⁻⁻ production was not attenuated in neutropenic I/R mice vs. sham mice or significantly affected by the neutrophil NAD(P)H oxidase inhibitor MFH244 during I/R, suggesting that TNF-α expression/production is not dependent on neutrophil activation.

I/R increased MPO activity, xanthine oxidase activity, and NAD(P)H oxidase activity. We have determined the influx of inflammatory cells into the myocardium by measuring MPO activity (Fig. 5A). In WT mice, I/R increased MPO activity compared with sham mice, and administration of anti-TNF-α...
before reperfusion reduced the MPO activity in I/R mice to the level observed in the sham group.

Xanthine oxidase activity and NAD(P)H oxidase activity from isolated coronary arterioles were elevated in I/R compared with sham. The treatment of anti-TNF-α, or allopurinol, or apocynin did not affect xanthine oxidase activity or NAD(P)H oxidase activity in sham (data not shown), but attenuated the activity of xanthine oxidase and NAD(P)H oxidase in I/R mice (Fig. 5, B and C). This suggests that TNF-α is expressed upstream from xanthine oxidase and NAD(P)H oxidase.

![Fig. 2. Dual fluorescence combining TNF-α with markers for endothelial cells (vWF) and vascular smooth muscle (α-actin) with the use of specific antibodies, followed by fluorescent-labeled secondary antibodies. A, B, and C: dual labeling of TNF-α (red) and α-actin (green) in sham control (CTL) mouse heart tissue. D, E, and F: dual labeling of TNF-α (red) and α-actin (green) in I/R mouse heart tissue. The pink arrow shows the staining of TNF-α (red), and the blue arrow shows the colocalization of TNF-α and vascular smooth muscle cells (yellow). G, H, and J: dual labeling of TNF-α (red) and vWF (green) in I/R mouse heart tissue. The white arrows in I and K show the specific vWF staining with absence of TNF-α staining. L and M: negative CTL. The purple arrows show an absence of staining in vessels with the secondary antibodies. N shows nuclear staining with 4,6-diamidino-2-phenylindole (blue) in I/R mouse heart tissue. Magnification ×40. Data shown are representative of 4 separate experiments.]

![Fig. 3. Isolated mice coronary arterioles from CTL mice (without I/R) dilated in a concentration-dependent manner to ACh (n = 10). A: neutralizing antibodies to TNF-α (1.6 mg/per mouse iv, before reperfusion) partially restored ACh-induced coronary arteriolar dilation in murine I/R, but anti-TNF-α did not affect ACh-induced coronary arteriolar dilation in murine sham (n = 4). B: administration of xanthine oxidase inhibitor allopurinol or NAD(P)H oxidase inhibitor apocynin before reperfusion partially maintained vasodilation to ACh in I/R. Furthermore, allopurinol or apocynin did not affect ACh-induced vasodilation in sham groups. However, the combination of allopurinol and apocynin (apocynin) had a greater protective effect than that seen with either agent alone. C: Interestingly, I/R mice rendered neutropenic or neutrophil NAD(P)H oxidase inhibitor MFH244 did not affect ACh-induced vasodilation in I/R groups. Values are means ± SD; n = 7. *P < 0.05 vs. sham mice; #P < 0.05 vs. I/R.]

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oxidase production, and TNF-α signaling affects the generation of xanthine oxidase and NAD(P)H oxidase.

I/R-induced ONOO\(^{-}\) (N-Tyr) production in coronary arterioles. Peroxynitrite (ONOO\(^{-}\)) has a very short half-life, so we measured the index of its formation, N-Tyr, by performing Western blot and immunohistochemical analyses. Western blot (Fig. 6A) and immunostaining (Fig. 6B) analysis for N-Tyr in homogenates from sham, I/R, and I/R mice treated with anti-TNF-α were reduced to sham control levels by anti-TNF-α. Our results in Fig. 6 were presented as fold change of N-Tyr in I/R mice normalized to internal control (β-actin), compared with sham mice (defined as 100 for sham control; data not shown).

**DISCUSSION**

Our major finding is that antibody neutralization of TNF-α after ischemia, but before reperfusion, ameliorated the consequences of I/R injury in the heart. Specifically, neutralization of TNF-α 1) prevented coronary endothelial dysfunction during myocardial I/R injury; 2) diminished enhanced expression of TNF-α (mRNA and protein) that occurs subsequent to I/R injury; 3) minimized invasion of macrophages; and 4) reduced ROS generation, xanthine oxidase activity, NAD(P)H oxidase activity, and ONOO\(^{-}\) production (N-Tyr) during I/R. Importantly, blockade of xanthine oxidase and NAD(P)H oxidase mimicked the actions of anti-TNF-α on \(O_2^{•−}\) production and endothelial function. Moreover, neither endothelial function nor \(O_2^{•−}\) generation during I/R was significantly affected in neutropenic animals, or by the neutrophil NAD(P)H oxidase inhibitor MFH244, suggesting that TNF-α is independent of neutrophil activation. Taken together, our results suggest that blockade of the inflammatory cytokine, TNF-α, exerts a therapeutic effect on the consequences of reperfusion injury, even when the neutralization occurs after ischemia.

**Cellular source of TNF-α expression and the role of TNF-α in I/R injury.** The microcirculatory changes produced by I/R injury are very similar to those associated with inflammatory processes, i.e., vasodilation, tissue invasion by inflammatory cells, and increased microvascular permeability (9). Thus it is appropriate to ask to what extent the measured increase in clearance really represents microvascular injury. The phenomenon may simply be an inflammatory response, which is a normal component of the immune defense reaction (9). TNF-α, as an important factor in I/R, is synthesized during the period of ischemia, and other organs also contribute to postischemic circulating TNF-α levels (27, 28). Circulating and cardiac TNF-α concentrations increase in response to myocardial I/R within minutes, most likely by release from macrophages, monocytes, and mast cells (3, 30). In native coronary vessels, TNF-α contributes to the progression from stable to unstable plaques by augmenting the local inflammatory response via

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ROLE OF TNF-α DURING REPERFUSION INJURY

**Fig. 6.** A typical nitrotyrosine (N-Tyr) blot shows bands migrating at 30 and 50 kDa (A, see arrows), and N-Tyr expression is significantly higher in I/R compared with the CTL and anti-TNF-α groups (B and C). Values are means ± SE; n = 3. *P < 0.05 vs. sham mice. #P < 0.05 vs. I/R mice. D–F: immunohistochemistry for N-Tyr, a marker of peroxynitrite formation, of representative sections of coronary arterioles from sham mice (D), I/R mice (E), and I/R + anti-TNF-α (before reperfusion) (F) shows chemiluminescence observed. All images were taken at ×40 magnification.

multiple effects (5, 30). TNF-α is expressed at low concentrations in the healthy heart, and such TNF-α is mainly located in the vascular endothelium. Left ventricular function as well as prognosis of heart failure patients are not improved following administration of TNF-α antibodies (25, 30). Our understanding of TNF-α and TNF-α receptors, especially with respect to structure-function relationships and their pathophysiological role in vascular dysfunction, is still in its infancy. TNF-α antagonist (soluble receptors) has been shown to lack benefits on the rate of death or hospitalization in patients with chronic heart failure (4, 6, 12, 15, 21, 24). This indicates the use of this treatment regimen is ineffective at this late stage of disease, but there have yet to be trials using TNF-α antagonists at earlier points in the development of vascular disease. If intervention is applied earlier, TNF-α antagonists may be more effective in the prevention of cardiovascular diseases. Moreover, there are many trials using TNF-α antagonists in vascular complications of inflammatory diseases, such as rheumatoid arthritis and Crohn’s disease, which suggest profound beneficial effects from anti-TNF-α treatment in improving vascular function (4, 6, 12, 15, 21, 24). However, there are very few trials applying anti-TNF-α treatment in vascular diseases and cardiovascular disorders related to Type 2 diabetes and metabolic syndrome.

Ischemic injury can be mimicked by TNF-α (18). Myocardial ischemia causes heart injury that is characterized by an increase in circulating TNF-α, the local production of O$_2^-$, the loss of coronary vasodilation in response to agents that release endothelial cell relaxation factor, and cardiac tissue damage (17). TNF-α expression in a model of partial coronary embolization can induce frank ischemia and induce dysfunction (7, 29). Our results show an increase in TNF-α expression (mRNA and protein) in I/R consistent with Lefer et al. (18), indicating this cytokine may have a key role in reperfusion injury. Our laboratory’s previous studies also show that plasma concentration of TNF-α was about sixfold increased in I/R vs. sham control mice (32). Monocyte chemoattractant protein-1 incites the migration of monocytes to the regions of formation and resorption bone, stimulating the production of proinflammatory cytokines (TNF, IL-6, IL-1) (1). TNF-α is one of the initiators of a cytokine cascade and may be one of the genes that are induced very quickly following I/R (16). The cell type expressing TNF-α may be related to infiltrating cells, such as macrophages and mast cells. In the normal myocardium, there are very few macrophages, but following I/R their numbers increase dramatically (13, 32). The adhesion and diapedesis of macrophages occur via alterations in endothelial cell functions, indicating failure of the normal barrier function that prevents these cells from migrating into the tissue.

In our study, the expression of TNF-α after I/R occurs primarily in the vascular wall and is colocalized with smooth muscle cells, mast cells, and macrophages in I/R in WT mice. These results indicate that the increased levels of TNF-α in the heart in I/R may be due, in part, to an increase of inflammatory cell migration into the vessels affecting vascular function. I/R increased the MPO staining, showing that MPO was expressed in I/R injury (likely due to infiltrating neutrophils). This supports our laboratory’s previous studies (14) showing that I/R-induced coronary endothelial dysfunction is, in part, mediated by TNF-α initiating inflammatory cell migration in I/R. However, the increasing MPO activity may not reach threshold levels needed to induce endothelial dysfunction, since neutropenia or MFH244 did not have overt significant effects on endothelial dysfunction.

Preservation of endothelial function will reduce the influx of inflammatory cells and maintain vasomotor control mechanisms. We determined the influx of inflammatory cells into the myocardium by using immunohistochemistry and by measuring MPO activity in the genetic models. The rationale for this measurement is that compromised endothelial function is associated with inflammatory cells, as reflected by migration of such cells through the endothelium and into the cardiac tissue. Vascular disease is frequently characterized by endothelial dysfunction (20), but patients with severe vascular disease show varying degrees of endothelial cell-dependent dilation. Moreover, the endothelial dysfunction that occurs with vascular disease in patients is typically not absolute, i.e., abrogated response rather than complete absence. Endothelial function is paramount in maintaining the endothelium against invasion of inflammatory cells, which contribute to reperfusion injury (13). Our MPO activity results support the idea that TNF-α plays a key role in endothelial dysfunction in reperfusion-induced injury.
The role of superoxide derived from xanthine oxidase and/or NAD(P)H oxidase in coronary vascular function in I/R. Some injurious effects of ROS occur in vivo during the first few minutes of reperfusion (8). This may explain why therapeutic benefits occurred when allopurinol or apocynin or both are administered at reperfusion. Anti-TNF-α reduces, but does not eliminate, the deleterious effects that occur with the production of this cytokine (ROS generation and inflammatory cell invasion) over the course of minutes following I/R. This could be a matter of timing. We administered the ROS scavenger and anti-TNF-α immediately before reperfusion to devise a more clinically relevant paradigm in which one can envision administration of anti-TNF-α during recanalization procedures or following bypass. Our laboratory’s previous study examined the use of ROS scavengers only in vitro, and the deleterious effects of ROS may have already occurred in vivo during the first few minutes of reperfusion (32). To prevent this, therapeutic benefits may occur when blockade of ROS is administered at reperfusion. Our present data show administration of inhibitors for xanthine oxidase and NAD(P)H oxidase immediately before reperfusion remediated, I/R-induced endothelial dysfunction in WT mice. Interestingly, Fig. 6A also shows the combination of allopurinol and apocynin did afford more “protection” than that seen with either agent alone, which indicates these two sources are independently involved in endothelial dysfunction at some threshold for injury. Although there are multiple intracellular sources for formation of oxygen free radicals, our results show the major enzymes activated by TNF-α during I/R are xanthine oxidase and NAD(P)H oxidase. The increased MPO produced by I/R raises the possibility that neutrophil-derived NAD(P)H oxidase and O$_2^-$ contribute to the observed responses. Our EPR results show that O$_2^-$ production was not attenuated in neutropenic (22) I/R mice or affected by the neutrophil NAD(P)H oxidase inhibitor, MFH244, suggesting that TNF-α is not working through neutrophil activation. However, because MPO activity is elevated in I/R and reduced by anti-TNF, the generation of hypochlorous acid may contribute to the endothelial injury.

Our data suggest that ONOO$^-$ is formed in conjunction with I/R-induced endothelial dysfunction; we infer the formation of ONOO$^-$ from the presence of N-Tyr residues. ONOO$^-$ might be another secondary mediator responsible for impaired vasodilation to ACh after I/R, but our results do not provide a definitive cause and effect for this ROS in the injury. Superoxide generated by vascular NAD(P)H oxidase, with subsequent formation of ONOO$^-$, mediates coronary endothelial dysfunction. These studies may be applicable in many clinical situations, such as recovery from bypass surgery, thrombosis, and other types of recanalization procedures. It is becoming more evident from the clinical literature that post-recanalization complications are clearly related (indirectly) to flow (and thus microvascular function). Thus if we can determine ways to maintain the integrity of microvascular control mechanisms, the incidence of complications may be targeted and reduced.

In conclusion, our results demonstrate that the endothelial dysfunction occurring subsequent to I/R injury is initiated by TNF-α, which induces activation of xanthine oxidase and NAD(P)H oxidase to produce O$_2^-$/ONOO$. Our results advance our understanding of the role of TNF-α in myocardial I/R injury by causally linking TNF-α expression to endothelial injury induced by myocardial I/R. Our present study shows that TNF-α plays a key role in endothelial dysfunction in perfusion-induced injury and is a promising target for therapies designed to preserve endothelial function critical to myocardial survival and recovery following I/R.

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REFERENCES


