TNF-α Contributes to Endothelial Dysfunction in Ischemia/Reperfusion Injury

Cuihua Zhang, Xiangbin Xu, Barry J. Potter, Wei Wang, Lih Kuo, Lloyd Michael, Gregory J. Bagby, William M. Chilian

Background—Despite the importance of endothelial function for coronary regulation, there is little information and virtually no consensus about the causal mechanisms of endothelial dysfunction in myocardial ischemia/reperfusion (I/R) injury. Because tumor necrosis factor-α (TNF-α) is reportedly expressed during ischemia and can induce vascular inflammation leading to endothelial dysfunction, we hypothesized that this inflammatory cytokine may play a pivotal role in I/R injury-induced coronary endothelial dysfunction.

Methods and Results—To test this hypothesis, we used a murine model of I/R (30 minutes/90 minutes) in conjunction with neutralizing antibodies to block the actions of TNF-α. TNF-α expression was increased 4-fold after I/R. To determine whether TNF-α abrogates endothelial function after I/R, we assessed endothelial-dependent (ACh) and endothelial-independent (SNP) vasodilation. In sham controls, ACh induced dose-dependent vasodilation that was blocked by the nitric oxide synthase (NOS) inhibitor L-NMMA (10 μmol/L), suggesting a key role for NO. In the I/R group, dilation to ACh was blunted, but SNP-induced dilation was preserved. Subsequent incubation of vessels with the superoxide (O2·−) scavenger (TEMPOL), or with the inhibitors of xanthine oxidase (allopurinol, oxypurinol), or previous administration of anti-TNF-α restored endothelium-dependent dilation in the I/R group and reduced I/R-stimulated O2·− production in arteriolar endothelial cells. Activation of xanthine oxidase with I/R was prevented by allopurinol or anti–TNF-α.

Conclusions—These results suggest that myocardial I/R initiates expression of TNF-α, which induces activation of xanthine oxidase and production of O2·−, leading to coronary endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2006;26:475-480.)

Key Words: coronary artery disease, endothelial function, nitric oxide, microcirculation, reactive oxygen species
The purpose of our study was to determine the role of TNF-α in endothelial injury during I/R has not been previously proposed. The mechanism by which TNF-α may induce injury appears to be the production of O$_2^-$, but linking TNF-α expression to O$_2^-$ production and endothelial dysfunction in endothelial injury during I/R has not been previously proposed. The purpose of our study was to determine the role of TNF-α in endothelial dysfunction during I/R injury by studying NO-mediated vasodilation to ACh in isolated and pressurized coronary arterioles, O$_2^-$ production, activation of xanthine oxidase, and expression of TNF-α with and without neutralizing antibodies to TNF-α.

**Methods**

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**Data Analysis**

At the end of each experiment, the vessel was relaxed with 100 μmol/L SNP and expressed as a percentage of maximal dilation. Data are presented as mean±SEM. Statistical comparisons of vasomotor responses under various treatments were performed with 2-way ANOVA and intergroup differences were tested with Bonferroni inequality. Significance was accepted at P<0.05.

**Results**

**I/R Increased TNF-α mRNA Expression and Plasma Concentration of TNF-α in Murine Coronary Arterioles**

Figure 1A shows the expression of TNF-α mRNA (204 bp) in left ventricular coronary arterioles of sham and I/R groups; expression of TNF-α was increased >4-fold after I/R. Figure 1B shows the inflammatory cytokine TNF-α is significantly increased after I/R (n=4), but anti-TNF-α attenuates the concentration of free TNF-α in I/R because the bulk of the circulating cytokine is bound to the antibody. However, plasma concentration of IL-6, IL-8, and IFN-γ are not increased after I/R (data not shown). Our studies indicate that after I/R, levels of TNF-α were the only cytokines to increase (P<0.05).

**Effects of I/R on NO-Mediated Vasodilation to ACh**

Isolated murine coronary arterioles from control and sham animals dilated dose-dependently to the endothelium-dependent agonist, ACh (Figure 2A). Administration of the nitric oxide synthase (NOS) inhibitor L-NMMA (10 μmol/L) reduced the vasodilatory responses to ACh compared with controls (Figure 2A). In a similar manner as L-NMMA, ACh-induced vasodilation was impaired after I/R (30 minutes/90 minutes). Function of smooth muscle was preserved, because SNP-induced vasodilation was equivalent in both groups (Figure 2B).

**Role of TNF-α in I/R-induced Vascular Dysfunction**

Neutralizing antibodies to TNF-α (I.P. 0.1 mg/mouse containing 16 mg protein/mL, administered 3 hours before initiating I/R) maintained NO-mediated coronary arterial dilation after I/R, but administration of nonimmune IgG did not (intrapерitoneal 0.1 mg/mouse containing 16 mg protein/mL, administered 3 hours before initiating I/R) (Figure 3A). Anti-TNF-α did not affect dilation in the sham group. We administered TNF-α (1 ng/mL, 60 minutes) and assessed responses of microvessels to ACh to show the acute administration of TNF-α could mimic the responses of I/R. ACh-induced dilation was significantly blunted by TNF-α or L-NMMA, but combined treatment (TNF-α plus L-NMMA) did not induce further abrogation of endothelial dilation (Figure 3B).
Incubation of arterioles isolated from coronary arterioles of control mice hearts with TNF-α triggered a pronounced increase in fluorescent signals in both endothelial and smooth muscle layers, indicating augmented production of O₂·− caused by abrogated endothelium-dependent dilation. This impaired vascular endothelial function as defined by abrogated endothelium-dependent dilation. This impairment has been attributed to arginase, depletion of tetrahy-
drobiopterin,21 O₂⁻, cytokines, proteases, and lipid mediators.10 Endothelium-derived NO is an important endogenous vasodilator that regulates microvascular tone. The endothelial release of NO underlies the mechanism of coronary arteriolar dilation to ACh.22,23 The endothelial NO component was evident in the present study because L-NMMA attenuated the ACh-induced vasodilation. Consistent with our previous studies, we show here that ACh evokes dilation of coronary arterioles (probably by activation of endothelial NOS). In the present study, endothelium-dependent ACh-induced vasodilation in mouse coronary artery was impaired after I/R, whereas endothelium-independent SNP-induced vasodilation was normal, and these diminished responses were not further reduced by treatment with a NOS inhibitor, suggesting that I/R produces endothelial dysfunction in a murine model of reperfusion injury. I/R injury is known to induce endothelial dysfunction,21 and our results speak to a causative role for TNF-α because anti-TNF-α prevented endothelial dysfunction.

**The Role of TNF-α in Coronary Vascular Function in I/R Injury**

Endothelial dysfunction is an important early recurring phenomenon in virtually all forms of I/R injury, including a variety of circulatory shock states.10 The role of TNF-α in ischemic injury in the intact heart is controversial with some groups reporting beneficial effects,24,25 whereas others find a detrimental role.26,27 TNF-α clearly initiates expression of an

**Figure 5.** DHE-fluorescence imaging of O₂⁻ in coronary arterioles (A). DHE fluorescence was markedly elevated in both endothelial (arrow head) and vascular smooth muscle (arrow) cells of arteriolar sections after TNF-α treatment (1 ng/mL, 60 minutes, n=4) and I/R (n=4) compared with vehicle (sham). Apocynin and nonimmune IgG did not, but TEMPOL, allopurinol, and anti-TNF-α, markedly reduced the fluorescent signals (n=4). A. Data representative from 4 separate experiments. B. EPR analysis of O₂⁻ production in isolated coronary arterioles in WT (sham), I/R, and I/R plus anti-TNF-α mice. a, Representative EPR tracing of tissue lysates obtained from WT, I/R, and I/R plus anti-TNF-α mice. b, The actual concentrations of O₂⁻ normalized by protein concentration. n=4 (B). *P<0.05 vs sham; P<0.05 vs I/R.

**Figure 6.** Xanthine oxidase activity in mouse left ventricular coronary arterioles. I/R significantly increased xanthine oxidase activity compared with sham mice. After neutralization with anti-TNF-α, xanthine oxidase activity was decreased but is still higher than those in sham mice. Allopurinol abolished I/R-induced increase of xanthine oxidase activity. However, anti-TNF-α and allopurinol did not affect the xanthine oxidase activity in sham mice. Data represent mean±SEM, n=9. *P<0.05 vs sham. **P<0.05 vs I/R.
entire spectrum of inflammatory cytokines. Accordingly, we hypothesized that TNF-α expressed in the endothelium might play a pivotal role in the regulation of NO-mediated vasodilation by increasing O$_2^-$ production, thereby decreasing NO bioavailability. Although we did not unequivocally eliminate a role for other cytokines in I/R injury, our results show that IL-6, IL-8, and TNF-γ were not elevated. Nevertheless, we are compelled in confined our conclusions to the effect of TNF-α because we did not systematically study these other cytokines.

A critical issue about our interpretations pertains to the specificity of the anti-TNF-α. Within this context, we have made several observations that demonstrate specificity of the antibody. Although the antibody is polyclonal, it shows no cross-reactivity with other cytokines. The ability of the antibody to bind to a few other recombinant cytokines was tested to a limited degree and we found binding only to murine TNF-α. The antigen was recombinant murine TNF-α and therefore it is very unlikely that this IgG neutralizes other cytokines. We used nonimmune control IgG. Moreover, in an animal model of lipopolysaccharide (LPS)-induced shock, anti-TNF-α completely blocked increases in plasma TNF-α, because the antibody bound the cytokine. In previous published and unpublished studies, anti-TNF-α has not universally attenuated LPS-induced effects on pulmonary parameters. This means TNF-α is not solely responsible for LPS-induced effects, and our antibody does not block whatever it is that causes these effects. Thus, we believe with conviction that the antibody is specific for TNF-α, and thus our interpretations and conclusions about the pivotal role this cytokine plays in endothelial I/R injury are correct.

There is no previous report on the endogenous role of TNF-α in this respect to our knowledge, and it has been unclear whether TNF-α plays a direct role in endothelial dysfunction during I/R injury. In the present study, we documented that TNF-α is critical for the development of endothelial reperfusion injury. TNF-α expression was significantly increased in murine coronary arterioles after I/R injury; neutralizing antibodies to TNF-α restored NO-mediated coronary arteriolar dilation in the I/R group, but did not affect the endothelium-dependent vasodilation in sham controls. To further establish this deleterious role of TNF-α, we attempted to mimic the endothelial injury by incubating isolated arterioles with TNF-α. A 60-minute incubation of endothelium-intact coronary arterioles with TNF-α, which did not alter resting diameter, blunted the ACh-induced vasodilatory response. Thus, TNF-α under the conditions of our experimental protocols appears to have a deleterious effect on vascular function by inducing endothelial dysfunction.

One interesting observation was an increase in TNF-α expression (4-fold) and an increase in plasma concentration of TNF-α (6-fold) after a total of 120 minutes of I/R. Although, we are not sure of the cell type that is expressing TNF-α, a component of the signal may be related to infiltrating cells such a macrophages. If TNF-α is, as we and others suspect, one of the initiators of a cytokine cascade, then it may also be one of the genes that is induced very quickly after perturbation.

Roles of ROS and Xanthine Oxidase in Coronary Vascular Function in I/R Injury

Endothelial dysfunction appears to occur very early after reperfusion, signaled by the endothelial generation of a large burst of superoxide radicals. The decrease in endothelium-dependent dilation has been shown to occur soon after the generation of O$_2^-$ by the reperfused coronary endothelium, suggesting that endothelial generation of O$_2^-$ radicals acts as a trigger mechanism for endothelial dysfunction. O$_2^-$ also reduces the bioavailability of NO, which would compromise dilation to endothelium-dependent stimuli. Thus, it appears that impaired NO-dependent functions, eg, vasodilation, are the direct result of the overproduction of O$_2^-$ during the development of I/R injury. Although many studies document oxygen radical formation during I/R, there is a paucity of knowledge regarding mechanisms responsible for stimulating the production of O$_2^-$.

Our results extend this knowledge by demonstrating the role of TNF-α in I/R-induced endothelial injury via stimulation of xanthine oxidase to produce O$_2^-$ as shown by our observation that anti-TNF-α prevented I/R-induced xanthine oxidase activation and O$_2^-$ production.

Although there are multiple intracellular sources for formation of oxygen free radicals [eg, mitochondria, NAD(P)H oxidase, etc], our results support the idea that the major enzyme activated by TNF-α during I/R is xanthine oxidase. We are not sure that allopurinol, in vitro, is acting as a free radical scavenger; however, we can state with conviction, based on our new results, that allopurinol is inhibiting xanthine oxidase. Our results indicated that the pathway for TNF-α-induced endothelial dysfunction is mediated by activation of xanthine oxidase and the subsequent production of O$_2^-$- This idea is supported by the finding of Pereda et al, which shows that simultaneous inhibition of TNF-α production and xanthine oxidase activity greatly reduced local systemic inflammatory response in acute pancreatitis. Our study shows that I/R injury increased the xanthine oxidase activity, and antibody to TNF-α and xanthine oxidase inhibitor, allopurinol, separately attenuated the xanthine oxidase activity in I/R injury. In the majority of studies cited pertaining to reperfusion injury, elevations in O$_2^-$ production are thought to be a critical factor in this process. Our results demonstrate the production of TNF-α is essential in eliciting this oxidative stress. The present study indicates that I/R increases TNF-α, which stimulates endothelial generation of O$_2^-$ through activation of xanthine oxidase in the endothelium and contributes to the endothelial dysfunction. To our knowledge, this is the first functional study to link the mechanism(s) of I/R injury—in terms of endothelial dysfunction—to TNF-α, the subsequent activation of xanthine oxidase, and thus the production of O$_2^-$ in coronary arteriolar endothelium. One caveat: we studied the role of TNF-α in endothelial dysfunction only in an acute setting. The chronic endothelial injury that occurs for weeks after I/R may be the result of a different mechanism. The study of this effect deserves further investigation.

In conclusion, our results demonstrate that the endothelial dysfunction occurring subsequent to I/R injury is caused by TNF-α, which induces activation of xanthine oxidase and production of O$_2^-$ We speculate that many conditions with a
moderate elevation of TNF-α, eg, septicemia, acute myocardial infarction, chronic heart failure, atherosclerosis, viral myocarditis, and cardiac allograft rejection\(^\text{(18,30)}\) may potentially mitigate the role of NO in coronary flow regulation and thus contribute to further deterioration of cardiac function. Selective modulation of xanthine oxidase signaling may provide a novel therapeutic target to prevent or alleviate coronary diseases associated with TNF-α activation.

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**References**

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Methods

Mouse Model. The procedures followed were in accordance with approved guidelines set by the Laboratory Animal Care Committee at LSUHSC. To understand the pathophysiological alteration of ischemic heart disease and to assess the potential role of TNFα in I/R, 12- to 15-week-old, 25- to 35-g male wild type (WT) mice were used in this study (Purchased from The Jackson Laboratory: FVB/NJ - Stock Number: 001800).

Murine I/R. Our studies of ischemia/reperfusion (I/R) followed the procedures and protocol described previously.\textsuperscript{1,2} Briefly, WT mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (30mg/Kg body wt of mouse). Each animal was placed in a supine position with paws taped to an electrocardiogram (ECG) board (lead II) to measure S-T segment elevations during I/R. The chest was opened for the I/R procedure. The ligation to obtain 30 min of ischemia proceeded with an 8-0 Sugipro monofilament polypropylene suture with a tapered needle passed underneath the LAD ~ 1-3 mm from the tip of the left auricle. A 1 mm section of PE-10 tubing was placed on top of the vessel, and a knot tied on top of the tubing to occlude the LAD, and the chest wall was approximated and covered with a piece of moistened gauze to prevent desiccation. Reperfusion for 90 min was induced by cutting the knot with microscissors, allowing release of the occlusion and reperfusion of the formerly ischemic bed. A sham group was subjected to the same surgical interventions without preforming occlusions. The control group had no surgical interventions.

TNFα Expression by Real-time PCR.\textsuperscript{3} Total RNA was extracted from left ventricular coronary arterioles using Trizol reagent (Life Technologies Inc), and was processed directly to cDNA synthesis using the SuperScript™ III Reverse Transcriptase.
(Life Technologies Inc). The following primers were designed (primer 3 software) and synthesized (Qiagen): TNFα forward, 5’-ctgggacagtgacctggact-3’; reverse, 5’-gcacctcagggaagagtctg-3’ (204 bp, accession No. NM-013693). GAPDH forward, 5’-aactttggcattgtggaagg-3’; reverse, 5’-acacattgggggtaggaaca-3’ (223 bp, accession No. BC083080). cDNA was amplified using qRT-PCR Kit with SYBR® Green (Life Technologies Inc). The $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct=\text{C}_{\text{T-TNF}}-\text{C}_{\text{T-GAPDH}}$) was used to analyze the change of TNFα gene expression. The mean threshold cycle ($C_t$) values for both the target (TNFα) and internal control (GAPDH) genes were determined. Data are presented as fold change of transcripts for TNFα gene in I/R mice normalized to GAPDH, compared with Sham mice (defined as 1.0 fold).

**Measurement of Plasma TNFα.** Blood collected in heparinized syringes was from vena cava in the various groups of mice and centrifuged at 4°C, 2500 rpm for 10 min. TNFα was measured using BIO-Plex mouse 3-plex assay (Bio-Rad Laboratories, CA, USA). Conjugate beads were added to the microplate to assess the level of TNFα in plasma. Each sample and standard was added to the beads in the well. After incubation for 30 minutes, the microplate was washed three times with BIO-Plex wash buffer, then 25 µl of a biotinylated detection antibody will be added to each well for 30 minutes. TNFα concentrations (pg/ml) were automatically calculated by BIO-Plex Manager software using a standard curve derived from a recombinant cytokine standard.

**TNFα Neutralization.** The neutralizing antibodies to TNFα (anti-TNFα) used in these studies was IgG goat polyclonal antibody prepared to be essentially lipopolysaccharide free. At 12 weeks of age, all mice were administered either with
nonimmune IgG (control; I.P. 0.1 ml/mouse containing 16 mg protein/ml, three hours before initiating I/R) or the neutralizing antibodies to TNFα (anti-TNFα IgG. I.P. 0.1 mg/mouse containing 16 mg protein/ml, three hours prior to initiating I/R). This concentration was based on previous studies and sufficient to neutralize endogenous TNFα.4,5

**Functional Assessment of Isolated Coronary Arterioles.** The techniques for identification and isolation of coronary microvessels were described in detail previously.7 After I/R, the heart was excised and immediately placed in cold (4°C) saline solution. Each coronary arteriole (50-80 µm in internal diameter) was carefully isolated and then used in the functional and molecular studies described below. To determine the response of coronary arterioles to ACh, vessels were cannulated with glass micropipettes and pressurized to 60 cm H2O intraluminal pressure. The cannulated vessel was bathed in physiological salt solution PSS (mmol/L: NaCl 145.0, KCl 4.7, CaCl2 2.0, MgSO4 1.17, NaH2PO4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and Mops 3.0) containing bovine serum albumin (1%; Amersham) at 37°C. For functional studies, vessels were cannulated with glass micropipettes and pressurized to 60 cmH2O luminal pressure without flow. After developing a stable basal tone (i.e., spontaneous constriction to 60-70% of maximal diameter), the experimental interventions were performed. The concentration-diameter relationships for ACh (0.1 nmol/L to 10 µmol/L) and SNP (1 nmol/L to 1 µmol/L) were then established.

To determine whether TNFα was playing a role in endothelial injury following I/R, endothelial dependent and independent dilation was assessed in coronary arterioles from nonimmune IgG or anti-TNFα IgG treated mice. ACh was used as an activator of
endothelium-dependent NO-mediated vasodilation. To determine the role of TNFα and O$_2^\cdot^-$ anions in murine I/R, the above vasodilatory functions were examined in the presence of a membrane-permeable O$_2^\cdot^-$ dismutase mimetic, TEMPO (1 mmol/L, 60-minute incubation). The contribution of NAD(P)H oxidase, xanthine oxidase and mitochondrial respiratory chain in generating O$_2^\cdot^-$ in I/R were assessed by treating the vessels with an NAD(P)H oxidase inhibitor apocynin (100 µmol/L),6,9 a xanthine oxidase inhibitor allopurinol (10 µmol/L)6,10 or oxypurinol (100 µmol/L),11 or the mitochondrial respiratory chain inhibitor rotenone (1 µmol/L)9 for 60-minute incubation, separately. All drugs were administered extraluminally in these functional studies.

**Measurement of O$_2^\cdot^-$.

**A. Dihydroethidium (DHE):** The production of O$_2^\cdot^-$ was evaluated in coronary arterioles with the oxidative fluorescent dye DHE.12,13 Isolated mice coronary arterioles (50-80 µm in diameter) were incubated in PSS containing vehicle at 37°C for 60 min, followed by embedding the arterioles in OCT compound (Tissue-Tek) for cryostat sections. DHE fluorescence for O$_2^\cdot^-$ in both endothelial and smooth muscle layers of vessels was measured in the WT mice before and after I/R alone or with anti-TNFα, apocynin or allopurinol. The embedded arterioles in OCT compound (Tissue-Tek) were cut into sections 12-µm thick and placed on glass slides. DHE was applied to each tissue section on the slide, which was incubated in a light-protected humidified chamber at 37°C for 30 min and then sealed with coverslips. Images were obtained using a Nikon fluorescence microscope (605-nm long-pass filter). Control and experimental tissues were placed on the same slide and processed under the same conditions.
**B. Electron Paramagnetic Resonance (EPR) Spectroscopy.**

A 10% vessel homogenate was prepared in a 50 mmol/L phosphate buffer containing 0.01 mmol/L EDTA. The homogenate was then subjected to low-speed centrifugation (1000g) for 10 minutes to remove unbroken cells and debris. The supernatants containing 2 mmol/L CP-H (1-hydrox-3-carboxypryridine) were incubated for 30 min at 37°C and frozen quickly in liquid nitrogen. EPR spectroscopy was performed at room temperature using a Bruker EMX spectrometer and 1 mm diameter capillaries. The EPR spectrum setting were as follows: modulation amplitude 1.0 gauss, scan time 83 seconds, time constant 163 ms and microwave power 40 mW, field sweep 60 gauss, microwave frequency 9.78 GHz, receiver gain 5 x 10^3, center field 3485 gauss. Superoxide quantitation from the EPR spectra was determined by double integration of the peaks, with reference to a standard curve generated from horse radish peroxidase generation of the anion from standard solutions of hydrogen peroxide, using p-acetamidophenol as the co-substrate, then normalized by protein concentration.

**Acute TNFα Incubation with Coronary Arterioles.** The isolated coronary arterioles were subjected to stimulation by intraluminal treatment of vessels with TNFα (1 ng/mL; R&D Systems) for 60 minutes to determine whether TNFα can inhibit coronary arteriolar dilations mediated by NO. ACh was used as an activator of NO-mediated vasodilation. The contributions of the NO pathway in these vasodilations were examined by treating the vessels with the NOS inhibitor N^G^-monomethyl-L-arginine (L-NMMA, 10 μmol/L, 30-minute incubation). All drugs were administered extraluminally in these functional studies, except TNFα.
Xanthine Oxidase Activity Assay. \(^{15,16}\) Left ventricular coronary arterioles were isolated and homogenized in PBS containing protease inhibitors. Xanthine oxidase activity was determined using Amplex Red xanthine/xanthine oxidase assay [Molecular Probes). 50 µl supernatant was added to 50 µl working solution (100 µmol/L Amplex Red reagent, 0.4 U/mL HRP and 200 µmol/L xanthine in 1× Reaction Buffer (0.1 M Tris-HCl, pH 7.5)] and incubated at 30°C under scotophase. After 30 min, the absorbance was measured at 560 nm in a microplate reader (Bio-Rad). Meantime, the xanthine oxidase standard curve was determined. Xanthine oxidase activity was expressed as mU/mg protein. Protein concentration was determined using BCA Protein Assay (Pierce Biotechnology, Inc), using bovine serum albumin as a standard.

Chemicals. All drugs were obtained from Sigma, except as specifically stated. ACh, SNP, L-NMMA, TEMPOL, and apocynin were dissolved in PSS for functional studies and in PBS for fluorescence detection. Rotenone was dissolved in DMSO, allopurinol and oxypurinol were dissolved in water. These drugs were then diluted in PSS to obtain the desired final concentration. The final concentration of DMSO in the tissue bath was 0.03%. Vehicle control studies indicated that these final concentrations of solvent had no effect on the arteriolar function.

References:


