Endothelial nitric-oxide synthase (eNOS) is an important regulator of endothelial function and vascular tone in biological tissues. While endothelial dysfunction occurs following ischemia and has been attributed to altered NO formation, the biochemical basis for this dysfunction is unknown. Therefore, studies were performed to determine the effects of myocardial ischemia and reperfusion on eNOS in isolated rat hearts subjected to periods of global ischemia or ischemia followed by reperfusion. eNOS activity was assayed by L-[14C]arginine to L-[14C]citrulline conversion and alterations in the amount and distribution of eNOS determined by Western blotting and immunohistochemistry. While activity was preserved after 30 min of ischemia with a value of 1.1 ± 0.1 pmol × min⁻¹ × mg of protein⁻¹, it decreased by 77% after 60 min and became nearly undetectable after 120 min. Reperfusion resulted in only a partial restoration of activity. The decline in activity with ischemia was due, in part, to a loss of eNOS protein. Hemodynamic studies showed that the onset of impaired vascular reactivity paralleled the loss of functional eNOS. Subjecting isolated eNOS to conditions of acidosis, which occur during ischemia, followed by restoration of pH as occurs on reperfusion, caused a combination of reversible and irreversible loss of activity similar to that seen in ischemic and reperfused hearts. Thus, loss of endothelial function following ischemia is paralleled by a loss of eNOS activity due to a combination of pH-dependent denaturation and proteolysis.

Over the last decade it has been shown that the endothelium plays a critical role in the control of vascular tone (1). A labile vasodilating substance termed endothelium-derived relaxing factor was identified as nitric oxide (NO), which is synthesized by a calcium dependent nitric-oxide synthase (NOS) in endothelial cells (2–4). Subsequently, it was observed that ischemia causes impaired endothelial reactivity (5). In the heart it was observed that coronary artery occlusion, as occurs in heart attack, results in endothelial dysfunction. In both isolated vascular ring and in vivo models, endothelium-dependent vasodilation is markedly decreased after myocardial ischemia and reperfusion (6–8). Studies in humans have identified a similar decline in acetylcholine-induced vasodilation or even paradoxical vasoconstriction in areas adjacent to atherosclerotic plaques present in coronary arteries (9). Likewise, a diffuse vasomotor impairment in hypercholesterolemic and diabetic patients was seen showing that vascular dysfunction might occur in different pathological conditions (10, 11).

Because of the major pathophysiological significance of impaired endothelial reactivity following ischemia, there has been great interest in determining its underlying mechanisms. Earlier studies verified that while receptor mediated endothelial-dependent responses to acetylcholine as well as receptor-independent responses to calcium ionophore A23187 were lost, endothelium-independent agents such as the NO donor nitroprusside could elicit normal vasodilation in coronary rings of reperfused hearts (5, 6). These observations implied the site of inhibition of endothelium-dependent dilation to be distal to receptor-mediated events and proximal to the activation of the vascular smooth muscle, suggesting that impaired enzymatic synthesis of NO could be one of the mechanisms causing endothelial dysfunction. However, questions remain regarding the contribution of a dysfunctional NO synthetase pathway to the endothelial impairment of postischemic hearts. The exact alterations in eNOS activity and expression which occur during ischemia or following reperfusion are unknown. Furthermore, it is not known what processes during ischemia cause these alterations.

In the heart, the endothelial isoform of nitric-oxide synthase (eNOS or NOS III) present in the endothelium of coronary vessels and myocardium, normally accounts for most NO production (12, 13). Catalysis by eNOS involves the oxidation of one of the terminal guanidino nitrogens of l-arginine to yield NO plus l-citrulline (14). Since nitric-oxide synthases are relatively labile enzymes (15) whose function can be impaired by a variety of conditions (16), we hypothesized that the metabolic disorders associated with ischemia and reperfusion might also impair eNOS activity. Therefore, the purpose of this study was to characterize the alterations in eNOS that occur following myocardial ischemia, as well as the mechanisms that trigger this process and their significance in causing endothelial dysfunction.  

MATERIALS AND METHODS

Isolated Heart Perfusion—Female Sprague Dawley rats (250–300 g) were heparinized and anesthetized with intraperitoneal pentobarbital. The hearts were excised, the aorta cannulated, and retrograde perfusion initiated at a pressure of 80 mm Hg using Krebs bicarbonate buffer (17 mM glucose, 120 mM sodium chloride, 25 mM sodium bicarbonate, 2.5 mM calcium chloride, 0.5 mM EDTA, 5.9 mM potassium chloride, and 1.2 mM magnesium chloride) bubbled with 95% O₂ and 5% CO₂ gas at 37 °C, as described previously (17). Hearts were perfused for 15 min to allow functional stabilization and then subjected to the desired duration of 37 °C global ischemia or ischemia followed by reflow. For studies of endothelial reactivity, coronary flow was measured...
after 15 min of perfusion. The vasodilatory effect of histamine (10⁻⁵ M) was then tested to measure base-line endothelium-dependent relaxation. Hearts were then subjected to control perfusion or 30, 60, or 90 min of ischemia followed by 45 min of reperfusion, and the effect of histamine on coronary flow was reassessed followed by measurement of the effect of the eNOS inhibitor N-ε-arginine methyl ester (L-NAME, 1 mM).

**Heart Tissue Homogenate Preparation**—Hearts were immediately frozen in liquid nitrogen, finely ground, and suspended in 3 ml of ice-cold buffer consisting of 50 mM Tris, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 12 mM mercaptoethanol, and the protease inhibitor leupeptin (20 μg/ml). The suspension was homogenized and centrifuged at 100,000 × g for 60 min at 5 °C. The particulate fraction was subsequently washed in 3 ml of ice-cold buffer containing 1 mM KCl for 5 min and centrifuged at 100,000 × g for 30 min at 5 °C. The supernatant was discarded, and the pellet was rinsed several times with buffer to remove excess KCl. Finally, the pellet was resuspended in buffer containing calmodulin (350 mM) and tetrahydrobiopterin (10 μM) which are essential cofactors removed from the pellet by the KCl wash (18).

**eNOS Activity Measurements**—eNOS activity was measured from the conversion rate of L-[¹⁴C]arginine to L-[¹⁴C]citrulline (19) in heart tissue subcellular preparations. The reaction mixture contained 3.0 mM NADPH, 200 μM CaCl₂, 30 μM EDTA, 30 μM EGTA, 100 mM calmodulin, and 10 μM tetrahydrobiopterin in Tris buffer. The reaction was initiated by the addition of purified L-[¹⁴C]arginine (317 μCi/μmol) to produce a 10 μM final concentration and carried out for 8 min at 37 °C. The reaction was quenched with 3 ml of ice-cold stop buffer (20 mM HEPEs and 2 mM EDTA, pH 5.5). Experiments were also performed in the presence of either EGTA (5.0 mM) or L-NAME (250 μM). L-[¹⁴C]Citrulline content was determined by liquid scintillation counting after separation from the reaction mixture by passage through a column of the cation exchange resin Dowex AG 50WX-8 (500 μl of the Na⁺ form). Samples of buffer containing L-[¹⁴C]arginine in the absence of heart tissue were added to the Dowex resin column to determine background counts, which were subtracted from all other measurements. eNOS activity was determined by subtracting total counts from L-NAME blocked counts and normalized for protein content (measured by the Bradford method) and conversion time. All activity detected was calcium-dependent and blocked by EGTA, confirming that it was from eNOS.

For studies of the pH dependence of enzyme denaturation, particulate fractions of heart homogenates were mixed with solutions of potassium phosphatate buffer producing different final pH values, and eNOS activity was assayed. To simulate pH changes that occur in ischemic and reperfused hearts, pellets were incubated with phosphatase buffers at pH 5.5 for 1.5, or 10 min alone or followed by restoration of pH to 7.4 with NaOH. eNOS activity was measured at each time point either immediately after renormalization of pH or after 10 min.

**Western Blotting**—Control, ischemic, and reperfused heart homogenates were aliquoted in 100-μg samples into 62.5 mM Tris-HCl buffer, pH 6.8, containing 25% glycerol, 2% SDS, and 5% β-mercaptoethanol, followed by heat denaturation at 96 °C for 5 min. Samples were loaded onto a 7.5% SDS-polyacrylamide gel, and electrophoresis was carried out for 30 min at 200 V. Proteins were transferred to a nitrocellulose membrane and blocked with 5% milk solution prepared in Tris-buffered saline with 0.05% (v/v) Tween 20. Rabbit polyclonal primary antibodies (1:500 dilution) raised against the polypeptides 1030–1209 of human eNOS were incubated with the membranes overnight at 4 °C. Then, biotinylated goat anti-rabbit secondary antibodies were incubated with the membranes for 2 h at room temperature followed by incubation with the streptavidin–biotinylated alkaline phosphatase complex. Membrane color was developed for approximately 10 min using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color development solution.

**Immunohistochemical Analysis**—Five-micrometer cryostat sections of heart tissue were fixed in aluminia-filtered acetone for 10 min and mounted on Histostik-coated slides. The slides were rinsed twice with Tris-buffered saline and incubated for 20 min in a humidity chamber with 0.1 mg/ml avidin followed by 0.05 mg/ml biotin and then quenched with 0.05% H₂O₂ (v/v). Sections were blocked with 0.5% dried milk and 1% normal goat serum for 10 min. Immunostaining was accomplished by sequential application of a mouse monoclonal antibody against the peptide 1030–1209 of the human eNOS (1:100 dilution), biotinylated goat anti-mouse Fc IgG, and streptavidin–alkaline phosphatase complex (phosphate buffer-3). The chromogen Fast Red substrate was used for labeling. Slides were counterstained with Mayer’s modified hematoxylin, dehydrated in increasing concentrations of ethanol, cleared with xylene, and coverslipped. As negative controls, heart sections were incubated with secondary and tertiary antibodies in the absence of the primary.

**RESULTS**

**Alterations in eNOS Activity during Myocardial Ischemia and Reperfusion**—eNOS activity was unchanged in hearts subjected to 30 min of ischemia (1.13 ± 0.11 pmol of L-citrulline/min × mg of protein) compared with controls (1.01 ± 0.10 pmol of L-citrulline/min × mg of protein) (Fig. 1). However, ischemic durations of longer than 30 min caused a sharp decline in eNOS catalytic function, which became almost undetectable after 120 min of global heart ischemia (0.06 ± 0.02 pmol of L-citrulline/min × mg of protein). These data show that, while in the early period of ischemia, normal enzyme activity persists; after prolonged ischemia of 60 min or more, eNOS activity is markedly impaired and may become a limiting factor for the synthesis of NO. All activity detected in these experiments was Ca⁺²-dependent, indicating no contribution of the inducible isoform of the nitric-oxide synthase to the global activity measured.

Upon reperfusion, a partial but significant recovery of eNOS activity was observed (Fig. 2). After 60 min of ischemia followed by 45 min of reperfusion, eNOS activity doubled when compared with end ischemic levels rising from a quarter to half of control levels. In addition, this recovery seemed to be more prominent in hearts subjected to shorter rather than longer periods of ischemia, suggesting that metabolic alterations that occur during ischemia may cause the loss of eNOS function.

**Effect of Ischemia and Reperfusion on the Expression and Localization of eNOS in the Heart**—To determine whether alterations in the amount of eNOS protein after ischemia and reperfusion account for the changes in enzyme activity, Western blots of eNOS were performed on the heart samples. A band of approximately 135 kDa compatible with eNOS was detected by the amplified alkaline phosphatase method. Progressive reduction of size and density of the eNOS band was noticeable after 45 min of reperfusion, eNOS activity doubled when compared with controls.
and 62%, respectively (Figs. 3A and 4A). However, no further alterations were seen after reperfusion (Figs. 3B and 4B). These data suggest that part of the loss in enzyme activity that occurs is due to protein degradation. This process is halted when flow is restored further, indicating that it is ischemia rather than reperfusion which results in enzyme dysfunction.

In control hearts a typical pattern of endothelial staining was seen with strong staining in arterioles, moderate staining in capillaries, and weak focal myocyte staining (Fig. 5). The changes in eNOS staining with ischemia paralleled those seen in the Western blots. After short periods of ischemia of 30 min or less no detectable change of eNOS staining in endothelial cells or myocytes was evident. In contrast, longer ischemic durations of 60 min induced a marked loss of staining in capillary endothelium and myocytes with a milder loss of staining in arterioles. These observations suggest that initial loss of eNOS occurs in capillary endothelial cells and myocytes followed by subsequent loss in arteriolar endothelium.

Measurement of Alterations in Endothelial-dependent Vascular Reactivity—To evaluate whether the loss of eNOS correlated with the onset of endothelial dysfunction, studies were performed to measure endothelial-dependent relaxation and endothelial-dependent constriction. Infusion of the endothelium-dependent vasodilator histamine ($10^{-5}$ M) in the coronary circulation of rat hearts at basal conditions induced a rapid increase in coronary flow. After the hearts were exposed to 30 min of ischemia followed by 45 min reflow, histamine-induced flow enhancement was only mildly blunted (22 ± 6%) compared with the control group (32 ± 3%; $p = \text{NS}$), whereas flow decrease imposed by L-NAME (1 mM) was still largely preserved (ischemia 30 ± 4% versus control 31 ± 2%). However, longer periods of ischemia were associated with a rapid decline of the endothelial response (Fig. 6). These results indicate that eNOS-associated endothelium-dependent response is just mildly im-

![Fig. 2. Effect of reperfusion on eNOS activity after 60, 90, and 120 min of ischemia. Data are expressed as percent of preischemic control levels with the solid bars showing the values obtained from measurements at end ischemia and the open bars from hearts after ischemia followed by 45 min of reperfusion. Significant recovery of eNOS activity was seen after reperfusion (*$p < 0.05$). The partial recovery of eNOS activity upon reperfusion indicates that part of the loss of enzyme function during ischemia is reversible. However, with increasing ischemia durations greater irreversible loss of eNOS activity is seen and the activity remains well below pre-ischemic levels.](image)

![Fig. 3. Immunoblotting of eNOS in control, ischemic, and reperfused hearts. A band of approximately 135 kDa from eNOS was detected in whole heart homogenates using the amplified alkaline phosphatase method. The amount of eNOS protein is preserved after 30 min of ischemia but drops after 60 or 90 min (A). No changes in the eNOS bands occurred following reperfusion suggesting that the levels of enzyme protein do not change on reperfusion (B). The 80-kDa band also seen in the gel is from alkaline phosphatase, AP, which is normally present in heart tissue.](image)

![Fig. 4. Quantitation of eNOS in Western blots of control, ischemic, and reperfused heart tissue. Each bar corresponds to the mean value of the densitometry data obtained from three experiments and standard error brackets are shown. Panel A shows that 60 min of ischemia induces a 38% reduction in the eNOS protein that further decreases to approximately 40% of control levels after 90 min. In panel B comparative results are shown after 60 or 90 min of ischemia or ischemia followed by 45 min of reperfusion. No significant additional change is seen after reperfusion.](image)
paired after short ischemic periods but becomes markedly impaired after prolonged ischemia. The time course of the onset of this endothelial dysfunction mirrored that of the loss of eNOS activity.

Mechanisms for the Loss of eNOS Activity—Because pH markedly decreases in ischemic hearts and may cause a loss of enzyme function, the effect of pH on eNOS activity was investigated. Heart particulate fractions that contain cardiac eNOS were titrated over the pH range from 7.4 to 5.5 and activity was measured and plotted as a function of pH (Fig. 7). Maximal eNOS catalytic function was observed at pH 7.4. Activity markedly decreased at pH values below 7.0 and at pH 5.5, enzyme function was almost completely lost. The observed titration curve of activity versus pH was accurately fit by the Henderson Hasselbalch equation with a pK value of 6.6.

To determine the effect of periods of acidosis similar to that occurring in the ischemic heart after 30 min of ischemia where pH falls to 5.5, as well as the effects of pH normalization as occurs on reperfusion, incubation of the partially purified eNOS was performed in phthalate buffer at pH 5.5 followed by normalization to pH 7.4 and immediate enzyme assay. Incuba-
tion in this acidic buffer caused a rapid loss of enzymatic activity, as indicated in Fig. 8. Exposing the enzyme for 1 min to this acidic pH resulted in a 29% decrease in eNOS function. Incubations as short as 10 min induced a decline of almost 80% of the enzymatic activity. However, when samples were incubated at pH 7.4 for 10 min after being exposed to pH 5.5 and then assayed for activity, a partial recovery simulating the in vivo response was detected. With enzyme subjected to short periods of acidosis of only 1 or 5 min, almost complete recovery of activity was seen within 10 min following renormalization of pH and with enzyme exposed to acidosis for longer periods of 10 min marked restoration of activity was seen with more than a 2-fold increase in eNOS activity from the value observed immediately after pH normalization. These results imply that the low pH associated with ischemia might play a role in the loss of eNOS activity whereas the recovery of pH to physiological levels during reperfusion may contribute to the partial restoration seen.

DISCUSSION

Loss of endothelium-dependent vasorelaxation occurs in hearts subjected to ischemia or ischemia followed by reperfusion (6–8, 20). Both receptor-mediated and nonreceptor-mediated endothelial responses are abolished, while endothelium-independent responses to vasodilators which directly form NO are maintained (5, 6). Therefore, it has been suggested that impaired NO’ synthase from eNOS is the cause of postischemic endothelial dysfunction, however, the mechanistic basis for this phenomenon was not known. While it was hypothesized that alterations in eNOS might occur, the presence, magnitude, time course, and nature of these alterations were not previously characterized. The data presented here provide the first determination of the alterations in eNOS protein and activity during myocardial ischemia and the role of these alterations in endothelial dysfunction.

The magnitude of NO’ formation from eNOS can be modulated by a number of factors including the level and activity of the enzyme; the concentration of the requisite substrates l-arginine, NADPH, and oxygen; and the levels of the cofactor tetrahydrobiopterin (16, 21). While all of these factors affect NO’ generation, the presence and activity of the enzyme is of paramount importance. Therefore, the present study focused on measuring the alterations in enzyme levels and function which occur during myocardial ischemia and reperfusion.

It was observed that short ischemic durations of 30 min or less did not affect enzyme activity as measured by l-arginine to l-citrulline conversion. Protein levels measured by Western blotting were also unchanged, and on immunohistochemistry no change in the amount or cellular localization of the enzyme was observed. However, with longer ischemic durations of 60 min or more a marked loss of activity was seen with 77% loss after 60 min, 84% loss after 90 min, and near complete loss after 120 min. This loss of activity was partially reversed after reperfusion, however, even after 45 min of reflow eNOS activity was still markedly decreased from preischemic values with residual 50, 35, and 20% levels seen in hearts which were subjected to 60, 90, or 120 min of ischemia, respectively. Thus, ischemia resulted in a marked time-dependent loss of enzyme activity with only partial restoration during subsequent reperfusion. Due to the acute nature of these isolated heart experiments, there was insufficient time for significant protein synthesis, therefore, these observations suggest that ischemia results in a combination of irreversible and reversible denaturation of eNOS.

Irreversible loss of activity could be due to degradation of eNOS protein. Western blotting demonstrated that while short ischemic times of 30 min caused no change in enzyme levels, longer ischemic times of 60 or 90 min, caused decreases of 38 and 62%, respectively. It has been previously reported that ischemia results in activation of cellular proteases (22). Therefore, it is probable that the loss of eNOS on Western blotting is due to protein degradation. This loss of eNOS protein was not reversed after reperfusion further demonstrating that the partial restoration of eNOS activity seen after reperfusion was not due to new protein synthesis. Ischemia results in marked intracellular acidification which can result in enzyme denaturation with a loss of enzyme activity. 31P NMR studies have previously demonstrated that after 30 min of global ischemia intramyocardial pH falls to a value of 5.5 (23). It has been previously reported that eNOS loses its

FIG. 8. Alterations in eNOS activity induced by periods of acidosis followed by pH normalization as occurs during ischemia and reperfusion. Heart tissue homogenates were subjected to 1, 5, or 10 min of incubation at pH 5.5, a value that occurs after 30 min of ischemia, followed by restoration to pH 7.4. Assays of eNOS activity were performed either immediately after pH normalization (solid bars) or 10 min after pH normalization (open bars). Time-dependent loss of activity was seen under acidic conditions, which was partially reversible after pH normalization. In tissue subjected to 10 min at pH 5.5, significantly higher recovery of activity was seen 10 min after pH normalization (*p < 0.01).

TABLE I

Correlation of ischemia-induced alterations in eNOS activity, eNOS protein, and vascular reactivity

| eNOS activity and protein values are expressed as percent of preischemic control values, while vasoreactivity values show either percent decrease in flow due to l-NAME (1 mM) or percent increase due to histamine (10⁻⁵ M). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 30 min          | 60 min          | 90 min          |
|                 | No reflow       | Reflow          | No reflow       | Reflow          | No reflow       | Reflow          |
| eNOS activity   | 100             | 112             | 109             | 23              | 50              | 16              | 35              |
| eNOS protein    | 100             | 114             | 114             | 62              | 66              | 38              | 35              |
| Vasoreactivity  |                 |                 |                 |                 |                 |                 |                 |
| l-NAME-induced vasoconstriction | 31      | 30              | 17              | 10              | 10              | 10              |                 |
| Histamine-induced vasodilation | 32      | 22              | 3               | 2               | 2               | 2               |                 |
catalytic function when the pH is lowered to this range (24). Experiments were performed to determine the precise effect of acidification on the activity of cardiac eNOS. We observed that loss of activity corresponded to the titration of a functional amino acid group with pK of 6.6. Since this pK value corresponds most closely to that expected for titration of the thiol group of cysteine and the critical heme center of eNOS contains an axially bound cysteine, it is possible that the loss of enzyme activity seen may correspond to titration of the heme bound cysteine. Alternatively it could represent titration of other cysteines on the enzyme or be due to effects on cofactor binding.

These data suggest that the loss of eNOS activity observed during ischemia could be explained by the following three-step process. First, in step 1, acidosis triggers the titration of a critical amino acid residue. Then, in step 2, this would lead to further altered protein conformation with a partially unfolded protein. Finally, in step 3, the unfolded protein conformation would then be susceptible to proteolytic degradation, leading to the irreversible loss of enzyme activity. This model would explain the stepwise loss of eNOS activity seen during ischemia that progresses from reversible loss of activity to irreversible loss of activity accompanied by degradation of the enzyme. It would also account for the progressive loss of enzyme activity and protein that occurs as a function of ischemic duration. The first two steps with pH-induced alteration in protein conformation could be potentially reversed upon renormalization of pH as occurs upon reperfusion; however, after proteolytic cleavage the loss of activity would be irreversible. The reversibility of steps 1 and 2 would explain why a partial restoration of activity can be seen following reperfusion, while step 3 explains the irreversible loss of enzyme which occurs.

The loss of enzyme activity closely correlated with the loss of endothelial vascular reactivity seen in these hearts. While only small changes in reactivity were seen after 30 min of ischemia, after 60 min, marked loss of vascular reactivity occurred. With administration of the NOS inhibitor L-NAME decreased flow is normally observed; however, after 60 min or 90 min of ischemia, this response was decreased by 45 or 68%, respectively, paralleling the loss of eNOS activity (Table 1). A marked loss of histamine-induced vasodilation was seen with a 90% decrease after 60 min of ischemia and a 95% decrease seen after 90 min of ischemia. The earlier loss of vasodilation at 30 min may indicate that, in addition to loss of enzyme, other alterations also occur that impair the vasodilatory response. Histamine induces vasodilation by increasing intracellular calcium concentrations, which in turn activate eNOS, a calcium dependent enzyme. During and following ischemia intracellular free calcium concentrations are elevated (25). This increase in basal calcium levels in the postischemic heart could result in further base-line activation of eNOS that would subsequently limit the magnitude of stimulated vasodilation to histamine. Previous direct electron paramagnetic resonance measurements of NO formation in postischemic hearts after 30 min of ischemia confirm that basal NO levels are increased after reperfusion compared with preischemic levels (26). Furthermore, it has been shown that after prolonged periods of ischemia increasing NO formation occurs due to enzyme independent formation from nitrate (23). Thus, while the loss in eNOS activity observed in the present study paralleled the observed loss of endothelial reactivity other mechanisms may also contribute to this process.

Prior studies in endothelial cell models have suggested other possible mechanisms that could also result in reduced enzymatic formation of NO. It has been suggested that substrate depletion of L-arginine or cussubstrate depletion of oxygen or NADPH could be important. It has been shown that endothelial derived relaxation is abolished by inhibitors of mitochondrial respiration (27) which result in decreased NADPH availability and that L-citrulline recycling to L-arginine by endothelial cells can be blocked causing the L-arginine pool to decrease (28). Similarly, tetrahydrobiopterin levels also regulate endothelial function (29). The formation of N-nitro-L-arginine-like endogenous substances that block eNOS has been described (30). While these other mechanisms could contribute to the alterations in vascular reactivity which occur during ischemia and reperfusion, they all require the presence of functional eNOS, so that loss of active enzyme would be of critical importance even if other alterations also occur. From our observations, these mechanisms could be important in the pathogenesis of altered vascular reactivity with relatively short ischemic durations, where eNOS activity is preserved.

It has been suggested that superoxide generated upon reperfusion may also result in rapid inactivation of NO (31, 34). Studies have demonstrated that superoxide dismutase can prevent postischemic injury (7, 32, 33). Recently, it has been demonstrated that superoxide and NO formed during early reperfusion react to form peroxynitrite, which in turn causes cellular injury (34). Since superoxide formation is increased only during the early period of reperfusion this mechanism could be involved in direct breakdown of NO during the early minutes of reperfusion. However, since the endothelial impairment persists in late reflow well after the burst of superoxide generation, it is clear that the observed alterations in endothelial function cannot be explained by simple scavenging of NO by superoxide. It has also been hypothesized that eNOS could be damaged by oxygen radicals generated during early reflow (35). However, we observe that enzyme function was impaired during ischemia and actually improved after reperfusion.

Thus, we observed that a marked progressive loss of eNOS activity occurred during ischemia with only partial restoration upon reperfusion. This loss of activity paralleled the loss of vascular reactivity observed, indicating that endothelial dysfunction occurred due to decreased functional eNOS. The loss of enzyme activity and protein observed, was best explained by acidosis-dependent denaturation, which was initially reversible, but was followed by proteolysis. These findings indicate that loss of eNOS activity and protein is a critical factor that results in endothelial dysfunction in the postischemic heart.

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