S-Nitroso Human Serum Albumin Treatment Reduces Ischemia/Reperfusion Injury in Skeletal Muscle via Nitric Oxide Release

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Background—Peroxynitrite generated from nitric oxide (NO) and superoxide (O2−) contributes to ischemia/reperfusion (I/R) injury. Feedback inhibition of endothelial NO synthase by NO may inhibit O2− production generated also by endothelial NO synthase at diminished local l-arginine concentrations accompanying I/R.

Methods and Results—During hindlimb I/R (2.5 hours/2 hours), in vivo NO was monitored continuously (porphyrinic sensor), and high-energy phosphates, reduced and oxidized glutathione (chromatography), and I/R injury were measured intermittently. Rabbits receiving human serum albumin (HSA) (controls) were compared with those receiving S-nitroso human serum albumin (S-NO-HSA) beginning 30 minutes before reperfusion for 1 hour or 30 minutes before ischemia for 3.5 hours (0.1 μmol · kg−1 · h−1). The onset of ischemia led to a rapid increase of NO from its basal level (50 ± 12 nmol/L) to 120 ± 20 and 220 ± 15 nmol/L in the control and S-NO-HSA–treated groups, respectively. In control animals, NO dropped below basal levels at the end of ischemia and to undetectable levels (<1 nmol/L) during reperfusion. In S-NO-HSA–treated animals, maximal NO levels never decreased below basal concentration and on reperfusion were 100 ± 15 nmol/L (S-NO-HSA preischemia group, 175 ± 15 nmol/L). NO supplementation by S-NO-HSA led to partial and in the preischemia group to total preservation of high-energy phosphates and glutathione status in reperfused muscle (eg, preischemia groups: ATP, 30.23 ± 5.02 μmol/g versus control, 15.75 ± 4.33 μmol/g, P < 0.0005; % oxidized glutathione, 4.49 ± 1.87% versus control, 22.84 ± 6.39%, P < 0.0001). S-NO-HSA treatment in all groups led to protection from vasoconstriction and reduced edema formation after reperfusion (eg, preischemia groups: interfiber area, 12.94 ± 1.36% versus control, 27.83 ± 1.95%, P < 0.00001).

Conclusions—Long-lasting release of NO by S-NO-HSA provides significant protection of skeletal muscle from I/R injury. (Circulation. 2002;105:3032-3038.)

Key Words: ischemia ■ reperfusion ■ nitric oxide ■ free radicals

Nitric oxide (NO) is seen as an essential substance of which basal levels must be maintained. Several pathological states, such as hypertension, atherosclerosis, stroke, diabetes, rheumatoid arthritis, and cancer, have been associated with abnormalities in NO generation.1 In addition, abnormally low NO concentrations (<1 nmol/L) have been measured in microvessels in vivo during ischemia/reperfusion (I/R) by use of porphyrinic microsensors.2

Reestablishing blood flow to ischemic tissues or organs (reperfusion) is an essential step in many surgical procedures.3 Especially after prolonged ischemia, however, reperfusion can lead to changes in vasomotility and an increase in microvascular permeability causing tissue reperfusion edema.3,4 These sequelae are constant features of I/R injury. The consequences of such an injury are frequently massive edema formation and tissue destruction.5,6

We hypothesize that the constitutive endothelial NO synthase (eNOS) plays a fundamental role in the pathogenesis of I/R injury. The onset of ischemia leads to elevated intracellular calcium ion concentrations mediated by increased levels of catecholamines. These increased calcium concentrations activate eNOS to generate NO and consequently reactive oxygen species and cytotoxic substances.7

These results strongly suggest a correlation between high initial NO production during ischemia followed by increasing release of O2− (decrease in net NO production) and increasing injury to the endothelium, constriction of vessels, and edema formation. The data also show that I/R injury is initiated by a
massive burst of NO production after ischemia that depletes local L-arginine concentrations, followed by high production of O$_2^-$ after reperfusion and consequently high production of peroxynitrite.2

Therefore, the strategy for using S-nitroso human serum albumin (S-NO-HSA) relies on the following deliberations: NO gradually released by S-NO-HSA can actively prevent the dysfunction of the endothelium by preserving eNOS. It is known that exogenous NO exhibits a negative feedback on the production of NO by eNOS (product inhibition of the enzyme).7 The exogenous NO released by S-NO-HSA can therefore decrease production of NO by eNOS. For the smaller turnover rate of eNOS, the local arginine concentration around the enzyme may be sufficient to minimize or even prevent derangement. The production of O$_2^-$ by the enzyme is at least reduced. The exogenous NO released by S-NO-HSA can also actively scavenge superoxide.

The study presented here investigates the role of S-NO-HSA in the reduction of I/R injury. In these experiments, continuous in vivo measurements of NO were correlated with changes in interfiber space (interstitial edema formation), microvascular changes (microvessel diameter alteration), and biochemical parameters (high-energy phosphates, glutathione status) measured intermittently as indicators of I/R injury in the skeletal muscles.

**Methods**

**Animal Preparation**

The studies were performed on adult male New Zealand White rabbits (2.8 to 3.5 kg; Charles River GmbH, Sulzfeld, Germany) in accordance with institutional guidelines. The animal preparation, housing, anesthesia, and lung ventilation parameters (Servo ventilator, 900C, Siemens) have been described in detail previously.2 Ventilator parameters were adjusted according to arterial blood gas values (pO$_2$, 155.8 ± 31.5, pCO$_2$, 37.3 ± 8.1, pH 7.40 ± 0.06) and oxygen saturation (ABL System, Radiometer). Arterial blood pressure was monitored with a pressure transducer in the carotid artery, and Ringer’s solution (0.2 mL·kg$^{-1}$·min$^{-1}$; Leopold Pharma GmbH) was infused via the auricular vein.

The animals were randomly divided into 5 groups and treated with S-NO-HSA or HSA (control). The treatment started 30 minutes before reperfusion or 30 minutes before ischemia (Figure 1).

Bilateral hindlimb ischemia was achieved by releasing the clamps and tourniquets and was confirmed by restoration of pulsatile blood flow in the femoral arteries as well as by the blood perfusion monitor.

At time points $t_0$=0 hours, $t_i$=2.5 hours, and $t_r$=2 hours, adductor magnus muscle tissue samples were taken from the right hindlimb for histomorphometric evaluation, and at time points $t_0$ and $t_r$, freeze-clamp biopsies were taken for biochemical analysis.

**S-NO-HSA Preparation**

HSA was processed to yield a maximal free thiol group at position Cys-34 (SH=0.8 mol/mol protein). Intermolecular disulfides (mixed disulfide) were disassembled before nitrosation. The starting material (20% HSA; Baxter) was reduced by mercaptoethanol (10- to 20-fold molar excess; buffer [mmol/L]: sodium phosphate 1, mmol/L EDTA 2, and sodium chloride 150, adjusted to pH 6.0 to 6.2 with HCl; 12 to 48 hours at 4°C under nitrogen) and purified by means of gel-permeation chromatography (TSK-HW40F; mobile phase, H$_2$O).

Thiol nitrosation was effected with sodium nitrite at a ratio of 1:1 to 1:1.5 of freely available thiol groups to nitrite in 0.2 mol/L HCl (pH 1.5 to 2.5) for 30 minutes at 25°C. After neutralization with 1 mol/L sodium hydroxide, S-NO-HSA was purified by gel-permeation chromatography (TSK-HW40F; mobile phase, H$_2$O and lyophilized. S-NO-HSA was dissolved and HSA was diluted with Ringer’s solution and continuously infused via a catheter in the jugular vein.

S-NO-HSA was measured in venous samples of the high-dose prereperfusion group by a postcolumn derivatization technique.8

**Biochemical Characterization**

**Preparation of NO Sensors for In Vivo Measurements**

NO release was monitored continuously in vivo in the wall of the femoral artery by a technique that used a porphyrinic microsensor.2 The porphyrinic microsensors were prepared by methods described in detail previously.9-11 Differential pulse voltammetry and amperometry were performed with a PAR model 273 voltammetric analyzer interfaced with an IBM 80486 computer with data acquisition and control software. Differential pulse voltammetry was used to measure the basal NO concentration, and amperometry was used to measure minute changes in NO concentrations with time (response time 0.1 ms; detection limit of the sensor 1 nmol/L). Linear calibration curves were constructed for each sensor from 2×10$^{-9}$ to 2×10$^{-7}$ mol/L NO before and after in vivo measurements with aliquots of saturated NO solution prepared as described.12

**Determination of High-Energy Phosphates**

This analytical method has been reported previously.13 In brief, the alterations were as follows. Separation was performed on a Hypersil ODS column (5 μm, 250 mm long×4 mm ID) with an AS-100
HRLC automatic sampling system (Bio-Rad), a 127 HPLC solvent module, and a diode array detector module (Beckman). Detector signals (absorbance at 214 nm for phosphocreatine [PCr] and 254 nm for adenine dinucleotides) were recorded with an AGC Personal Computer. System Gold (Beckman) was used as controller for data requisition and analysis.

Skeletal muscle tissue samples were freeze-clamped and stored in liquid nitrogen until further treatment. The weighed tissue (20 to 100 mg) was homogenized with 250 μL of 0.4 mol/L perchloric acid in a ball mill (Braun) precooled with liquid nitrogen. After thawing (4°C) and centrifugation (12 000 g at 70 °C) and centrifugation (12 000 rpm, 10 min), the supernatant and residual acid extract (glutathione determination) were stored (−28 °C) and neutralized with 12.5 L of 2 mol/L potassium carbonate. After centrifugation, the supernatant and residual acid extract (glutathione determination) were stored (−28 °C) until analysis. The pellets of the acid extract were dissolved in 1 mL of 0.1 mol/L sodium hydroxide until further treatment. The weighed tissue (20 to 100 mg) was homogenized with 250 μL of 0.4 mol/L perchloric acid in a ball mill (Braun) precooled with liquid nitrogen. After thawing (4°C) and centrifugation (12 000 g at 70 °C) and centrifugation (12 000 rpm, 10 min), the supernatant and residual acid extract (glutathione determination) were stored (−28 °C) until analysis. The pellets of the acid extract were dissolved in 1 mL of 0.1 mol/L sodium hydroxide and further diluted 1:10 with physiological saline for protein determination (BCA Protein Assay, Pierce).

**Determination of Reduced and Oxidized Glutathione**

The analyses were performed in principle according to a previously described method. In brief, samples were chromatographed on a Spherisorb S3ODS-2 column (3 μm, 125 mm long×4 mm ID) with a Rheodyne 7125 injector (Cotati), a PU-980 HPLC pump (Jasco), a pulsation damper (Shodex, model DP1), and a Coulochem 5100 A electrochemical detector (ESA) equipped with a 5020 guard cell (potential 0.4 V) and a 5011 analytical cell (first electrode potential, 0.9 to 1.2 V, gain 1, for reduced glutathione [GSH]; second electrode potential, 0.4 V) and a 5011 analytical cell (first electrode potential, 0.9 to 1.2 V, gain 10, for oxidized glutathione [GSSG]). The detector signals were collected via an analog interface (Rheodyne 7125) and a Coulochem 5100 A detector module, and a diode array detector module (Beckman). Detector signals (absorbance at 214 nm for phosphocreatine [PCr] and 254 nm for adenine dinucleotides) were recorded with an AGC Personal Computer. System Gold (Beckman) was used as controller for data requisition and analysis.

**Morphological Investigations**

Adductor magnus muscle tissue samples were immediately immersed for quick freezing at −70°C in 2-methylbutane (Uvasol; Merck) for 2 minutes. Then the samples were stored at −80°C until they were sectioned. Transverse cryosections 10 μm thick (Kryostat 1720, Leitz) were stained for actomyosin-ATPase activity at pH 4.3 and were examined by light microscopy (Axioskop, Zeiss) by an unbiased observer. The methods for determination of the percentage of muscle interfiber area and microvessel cross-sectional area have been described previously.2

**Statistical Analysis**

The mean±SD values are given (n=8; S-NO-HSA group: 1 μmol · kg⁻¹ · h⁻¹, n=7). Comparison between groups and between different time points were performed by blocked and unblocked ANOVA and paired and unpaired t tests (statistical software, SAS 1990).

**Results**

**NO Release**

Figures 2 and 3a show typical profiles of NO release in the experimental groups during I/R measured in vivo with a porphyrinic sensor placed in the wall of the femoral artery. A rapid increase of NO from its basal concentration (50 ± 12 nmol/L) was observed in all groups after clamping of the femoral artery. An increase in NO to 88 ± 20 nmol/L was detectable before clamping (Figure 3a) only in the group receiving S-NO-HSA 30 minutes before ischemia. Maximum values of NO reached after the onset of ischemia (peak, 15 to 20 minutes) were 140 ± 25 nmol/L (S-NO-HSA) and 120 ± 20 nmol/L (HSA) in the prerefusion groups. Then the concentration of NO decayed at a rate of 0.05 ± 0.02 nmol·L⁻¹·s⁻¹, falling below basal levels between 50 and 70 minutes after the onset of ischemia. After another 30 minutes, the values were significantly below the normal preischemic basal concentrations. During the first 5 minutes of reperfusion, a rapid decrease of NO concentration to or near the detection limit for GSSG, 20 pmol).

**Figure 2.** In vivo measurements of NO release in rabbit hindlimb with intervention during I/R in control group receiving HSA at a dose of 1.0 μmol·kg⁻¹·h⁻¹ (a) and treatment group receiving S-NO-HSA at a dose of 0.1 μmol·kg⁻¹·h⁻¹ (b). Continuous intravenous infusion of either HSA or S-NO-HSA started 30 minutes before reperfusion and was maintained for 1 hour.

**Figure 3.** In vivo measurements of NO release in rabbit hindlimb during pre-treatment with S-NO-HSA (0.1 μmol·kg⁻¹·h⁻¹ for 3.5 hours) starting 30 minutes before ischemia (a) and plasma concentration profile of S-NO-HSA in high-dose prerrefusion group (1.0 μmol·kg⁻¹·h⁻¹) measured in samples from distal interior caval vein (b).
limit (<1 nmol/L) was observed in the group receiving HSA (Figure 2a). This was also observable in the group of animals receiving HSA before ischemia. In these control groups, the levels of NO remained at these low to undetectable concentrations for 30 to 50 minutes and then increased toward the end of the experiment (2 hours of reperfusion) to maximal values of 15±10 nmol/L. In contrast, NO concentrations did not fall to undetectable levels after 5 minutes of reperfusion in the groups receiving S-NO-HSA (prereperfusion group, 15±10 nmol/L; preischemia group, 25±5 nmol/L). Maximal values on reperfusion were observed after 30 minutes (prereperfusion group, 100±15 nmol/L; preischemia group, 175±15 nmol/L) with no or little decline over the residual experimental period. The maximal values of NO reached during ischemia for the group receiving S-NO-HSA before ischemia was 220±15 nmol/L. This concentration decayed at a slower rate (0.03±0.01 nmol · L⁻¹ · s⁻¹) than the other groups (Figure 3a). Because of the detection limit of the technique, Figure 3b shows the plasma concentrations of S-NO-HSA measured only in the high-dose prereperfusion group. From these results, a plasma half-life of 16 minutes was determined for S-NO-HSA. A contribution of iNOS to the total NO production is minimal in the I/R model used in these studies. In the presence of iNOS (selective inhibitor, 1400 W), the NO production decreased by only 3%.

Microvessel Cross-Sectional Area
In all treatment groups with S-NO-HSA, microvessel constriction in the reperfused muscle was prevented (Figure 4). This is demonstrated by the unchanged microvessel cross-sectional area. No significant changes were observed either at the end of ischemia or after 2 hours of reperfusion compared with baseline values. In contrast, the control groups receiving HSA showed significant microvessel vasoconstriction in the reperfused muscle. Microvessel cross-sectional area was significantly reduced 2 hours after reperfusion (Figure 4a and 4b). No significant changes in microvessel size were seen in any group at the end of the ischemic period compared with baseline values. Illustration of the changes in microvessel size on transverse sections of adductor magnus muscle is shown in Figure 5.

Percent Muscle Interfiber Area
The interstitial edema was significantly reduced in the groups receiving S-NO-HSA before reperfusion (Figure 6a). This was evident from the decreased percent muscle interfiber area in the reperfused muscle in the treatment groups compared with the control. At the end of ischemia, all groups showed a slight increase in percent muscle interfiber area compared with baseline values (Figure 6).

Almost no edema was observed in the group receiving S-NO-HSA before ischemia (Figure 6b). The percent muscle interfiber area in the reperfused muscle was increased by 8.3%.

An illustration of typical stained transverse cryosections of the adductor magnus muscle is presented in Figure 7.
High-Energy Phosphates in Muscle Biopsies

Determination of high-energy phosphates in the reperfused muscle revealed that S-NO-HSA treatment preserves mitochondrial function. Compared with the control group (HSA 1.0 μmol · kg⁻¹ · h⁻¹), there was a significant preservation of PCr and ATP in skeletal muscle in both prereperfusion treatment groups (Figure 8a and 8b).

In the group receiving S-NO-HSA before ischemia, both the PCr and ATP values in the reperfused muscle did not differ from the corresponding baseline values, and they were highly significant compared with the control group (HSA, 0.1 μmol · kg⁻¹ · h⁻¹).

For more details see Figure 8c and 8d.

Glutathione Status in Muscle Biopsies

For the ratio determination, GSSG was calculated as GSH equivalents and determined for each muscle biopsy. It represents the percentage value of GSSG expressed as GSH. S-NO-HSA in both prereperfusion treatment groups significantly reduced the glutathione ratio in the reperfused muscle compared with the control group. In the preischemia group, we analyzed only the extracts of the biopsies from the reperfused muscles in the S-NO-HSA group. The value of 4.49 ± 1.87% is directly comparable to the previously determined baseline value (Figure 9).

Discussion

Data presented here clearly show that S-NO-HSA preserves the function of eNOS, stabilizes the basal production of NO, decreases production of oxidized species, and therefore has beneficial effects in reduction of I/R injury. NO concentration measurements reveal that infusion of S-NO-HSA (0.1 μmol · kg⁻¹ · h⁻¹) in the rabbit hindlimb model leads to a concentration in the wall of the femoral artery of ~100 nmol/L (prereperfusion group) or 175 nmol/L (preischemia group) in the reperfusion phase. This corresponds to 2 or 3.5 times the baseline value, respectively. These concentrations of NO are sufficient to reduce I/R injury. NO deficiency as a result of its consumption by O₂⁻, produced in high concentrations during ischemia and reperfusion, plays an important role in the pathophysiology of I/R injury. The mechanism by which S-NO-HSA as an exogenous NO donor can protect the dysfunction of the endothelium and prevent excessive O₂⁻ formation is based on prevention of eNOS derangement. The deranged NOS can produce both NO and O₂⁻.

NO generated by S-NO-HSA prevents eNOS derangement in prolonged ischemia. This may further prevent the formation of excessive local peroxynitrite concentrations and consequent cleavage products during the initial phase of reperfusion. Three of these cleavage products (hydroxyl free radical, nitrogen dioxide free radical, and nitronium cation) are among the most reactive and damaging species and may be major contributors to the severe I/R damage signaled by profound microvessel constriction and persistent interstitial edema.

It is evident from the concentration profiles in the control animals that there is a deficiency of NO at the end of ischemia and at the beginning of reperfusion, indicating a requirement for an NO donor. Despite the late intervention in the prereperfusion phase (onset of drug infusion after 2 hours of...
ischemia), both concentrations of S-NO-HSA significantly reduced reperfusion edema. The concentration gradient of NO achieved with both concentrations of S-NO-HSA from the arteries and microvessels via the endothelium to the smooth muscle was sufficient to prevent microvessel constriction. Blood flow was not hindered, and reperfusion damage to the tissue, as demonstrated by the morphometric results of the adductor magnus muscle, was significantly reduced 2 hours after ischemia. Pretreatment with S-NO-HSA, such as may be indicated in elective surgery, was even more beneficial. This could be because NO released by S-NO-HSA can counteract the consequences of the initial excessive NO production by eNOS or because NO concentrations are maintained above baseline for an essentially longer period in the ischemic region. This further protects the enzyme.

It is important to emphasize that treatment with 0.1 μmol · kg⁻¹ · h⁻¹ S-NO-HSA (ie, the lower concentration tested) achieved the same beneficial results as the high dose (1 μmol · kg⁻¹ · h⁻¹ S-NO-HSA) in the prereperfusion groups studied. No significant differences were observed between the S-NO-HSA and HSA groups in terms of mean arterial blood pressure and heart rate, but the high dose is in the range in which it can influence blood pressure on reperfusion. This is not the case with the low dose.

Determination of high-energy phosphates revealed that S-NO-HSA treatment preserved mitochondrial function. There was a significant preservation of PCr and ATP in skeletal muscle of all treatment groups, with no differentiation of baseline values in the pretreatment group.

Energy charge (EC), a crucial parameter reflecting the energy level of the energetic situation of the cell, was improved in the skeletal muscle with S-NO-HSA treatment. EC was calculated according to following formula: EC = (ATP + 0.5 ADP)/(AMP + ADP + ATP). All treatment groups revealed improvement in EC compared with the control groups.

In the pretreatment group, however, EC was directly comparable to the baseline values (S-NO-HSA reperfused muscle, EC = 0.923 ± 0.006; baseline, EC = 0.927 ± 0.006; control, EC = 0.858 ± 0.058). The ratio of protein to wet weight (expressed in % protein) reflecting the water content also confirmed the beneficial effects of S-NO-HSA treatment (pretreatment group, 13.53 ± 0.76% versus control, 11.40 ± 1.49%, P<0.01; baseline, 14.15 ± 1.06%) and is in accordance with the histomorphometric data.

The measurements of the glutathione status revealed a reduction of oxidative stress (radical formation; eg, O₂⁻) in the S-NO-HSA treatment groups. GSH is often seen as the first line of defense within the cell against tissue injury from metabolically generated (or administered) electrophiles. The concentration of GSH by far exceeds that of GSSG, which is effectively converted into GSH by the NADPH-utilizing enzyme glutathione reductase. As an intracellular reductant, it serves in the destruction of free radicals and hydrogen peroxide. This protection against oxygen toxicity results in the conversion of glutathione to glutathione disulfide. Therefore, the ratio of oxidized to reduced glutathione is an indicator of tissue oxidative stress. Data presented here clearly indicate that the glutathione ratio increased from the
basal level of 4.6% to 22.8% in the reperfused muscle of the control group. All S-NO-HSA treatment groups significantly reduced the glutathione ratio compared with the control group. Pretreatment did not even allow discrimination of baseline values, indicating maximum protection against oxidative stress.

In conclusion, the present study demonstrates a protective effect of S-NO-HSA treatment of skeletal muscle against I/R injury applied either before reperfusion or as pretreatment. The concentration of NO delivered to endothelial cells by this novel NO donor is in the range of physiological concentrations. Therefore, S-NO-HSA treatment does not affect blood pressure and can be a powerful tool in preventing or reducing ischemia-reperfusion injury in skeletal muscle.

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