S-Nitrosoglutathione induces formation of nitrosylmyoglobin in isolated hearts during cardioplegic ischemia – an electron spin resonance study

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Abstract Previously, it has been shown that •NO donor *S*-nitrosoglutathione (GSNO) improves the postischemic functional recovery in crystalloid buffer-perfused isolated rat hearts subjected to cardioplegic ischemia. Supplementation of cardioplegic solution with nitronyl nitroxide, a scavenger of •NO, antagonized this protective effect. Using low temperature ESR, we have detected nitrosylmyoglobin (MbNO) in rat hearts subjected to cardioplegic ischemia in the presence of GSNO (20– 200 μmol/l). During aerobic reperfusion MbNO signal intensity gradually decreased, but persisted for up to 30 min of aerobic reperfusion. We conclude that MbNO is an endogenous marker of •NO release in myocardial tissues. Implications of MbNO formation are discussed with respect to cardioprotection during ischemia- and reperfusion-induced myocardial injury.

Key words: Myocardium; *S*-Nitrosothiol; Nitrosylmyoglobin; ESR; Ischemia-reperfusion injury

1. Introduction

The current status of the role of nitric oxide (•NO) in cardiovascular diseases is controversial [1–5]. Several studies have shown that •NO donors protect the heart against ischemia/reperfusion injury in vitro and in vivo animal models [1–3]. In other studies [4,5], inhibitors of nitric oxide synthase were shown to be beneficial during myocardial ischemia. Using an isolated crystalloid buffer-perfused model, we have recently shown that supplementation of cardioplegic solution with *S*-nitrosoglutathione (GSNO), a physiologically-relevant •NO donor, improved post-ischemic functional recovery [6]. Intermediacy of •NO was inferred from the antagonistic effects of nitronyl nitroxide, an effective scavenger of free •NO [6,7].

Cardiac myocytes are enriched with myoglobin, which is thought to be involved in the intracellular transport and storage of oxygen [8]. Redox cycling of myoglobin has been linked to oxidative myocardial damage observed during ischemia and reperfusion [9,10]. •NO reacts fairly rapidly with heme-proteins ($k = 10^7\ M^{-1}\cdot s^{-1}$) [11]. Nitrosylmyoglobin (MbNO) formation has been shown to mitigate the oxidate damage induced by myoglobin [12–14]. Therefore, direct monitoring of MbNO formation and decay may be critical to understanding its role in myocardial injury.

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Abbreviations: ESR, electron spin resonance; GSNO, S-nitrosoglutathione; MbNO, nitrosylmyoglobin; KHB, Krebs-Henseleit buffer; Mb, myoglobin.

The objectives of this study are: (1) to directly demonstrate by low-temperature electron spin resonance (ESR) spectroscopy the formation of nitrosylmyoglobin in heart tissue; and (2) to investigate the effect of postischemic reperfusion on the formation of MbNO and free radicals in GSNO-treated hearts.

2. Materials and methods

2.1. Materials

The nitronyl nitroxide, 2-(*p*-trimethylammonium)-phenyl-4,4,5,5-tetramethyl imidazoline 3-oxide-1-oxyl, was synthesized according to published methods [6]. GSNO was prepared according to a procedure described by Hart [15]. The authentic MbNO was prepared from metmyoglobin (Sigma Chemical Co.) and Angeli's salt as described previously [16]. 'Baker analyzed' high purity reagents (JT Baker, Philipsburg, NJ) were used in perfusion media. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Isolated heart perfusion

Adult male Sprague–Dawley rats (300–350 g b.wt.), maintained on a standard diet, were used for this study. Rats were anesthetized with pentobarbital (50 mg/kg b.wt. i.p.) and heparinized via the left femoral vein (250 IU/kg). After 1 min, the heart was excised rapidly and placed in perfusion medium. Within 30 s, the aorta was attached to a stainless steel cannula. The pulmonary artery was incised to permit adequate coronary drainage, and the heart was perfused normothermically by the method of Langendorff at a perfusion pressure equivalent to 12 kPa (90 mmHg). The perfusion medium used was KHB with the following composition in mmol/l: NaCl 118.5, $NaHCO_3$ 25, KCl 4.8, $MgSO_4\cdot 7H_2O$ 1.2, KH_2PO_4 1.2; and glucose 11.1, in which the $CaCl_2\cdot 2H_2O$ content was reduced to 1.8 (pH 7.4 when gassed with 95% O_2 and 5% CO_2). During preparation of KHB, precautions were taken to prevent the precipitation of calcium by gassing the solution with 5% CO_2. St. Thomas' II solution was used as a cardioplegic solution with the following composition in mmol/l: NaCl 110, $NaHCO_3$ 10, KCl 16, $MgCl_2\cdot 6H_2O$ 16, and $CaCl_2\cdot 2H_2O$ 1.2 (pH was titrated to 7.8 using HCl).

2.3. Tissue preparation

After selected intervals of aerobic perfusion, ischemia, and reperfusion, as shown in section 2.5 (Fig. 1), hearts were freeze-clamped between stainless steel tongs previously cooled to the temperature of liquid nitrogen. Frozen ventricular tissue was then chopped under liquid nitrogen with a stainless steel spatula to produce small fragments (≃ 2 mm cubes). These fragments were immediately transferred to the lumen of a dewar flask that had been precooled to the temperature of liquid nitrogen. For ESR measurement at liquid helium temperatures, the frozen tissue was further chopped into smaller fragments (= 1 mm) and inserted into a 4 mm o.d. quartz tube immersed in liquid nitrogen. Samples prepared by this technique did not artifactually generate radical species [17]. Myocardial tissue homogenate was prepared in ice-cold phosphate-buffered saline (25 mmol/l, pH 7.4) using Brinkman homogenizer. Immediately after homogenization, tissue homogenate was transferred to ESR quartz tubes (4 mm o.d.) and frozen in liquid nitrogen.

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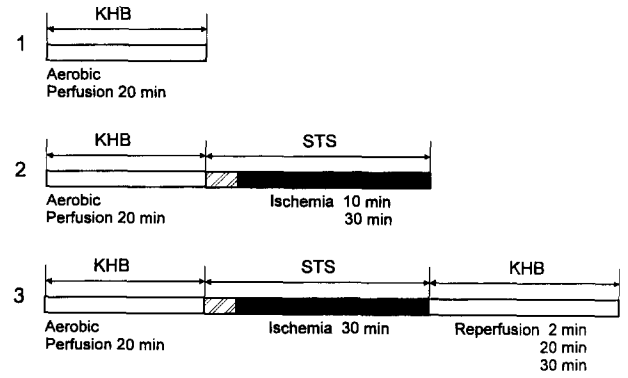

Fig. 1. Experimental protocols. Hatched area in 2 and 3 is 3 min perfusion with St. Thomas' II cardioplegic solution (STS) to arrest the heart before induction of ischemia. KHB = Krebs-Henseleit buffer.

2.4. ESR spectroscopy

ESR spectra from all samples were recorded at liquid nitrogen temperature with a Varian E-109 spectrometer at 9.5 GHz and 100 kHz field modulation. The measurements at 15 K were performed with a rectangular TE_{102} cavity from Varian. A water warming jacket was used to keep the cavity at a constant temperature. Sample temperatures were controlled by a Heli-Tran flow system (Allentown, PA) comprised of a transfer line and digital indicator/controller and a quartz insert dewar. Magnetic field measurements were determined by a using a Radiopan MJ-110 gaussmeter, and microwave frequency was measured by using an EiP 200 frequency counter. The g-values were estimated from measurement of magnetic field and microwave frequency after correcting for the position of the gaussmeter probe. All spectra were obtained by signal averaging (5 scans).

2.5. Experimental protocols

In protocol 1, hearts were perfused aerobically with KHB at 37°C for 20 min. In the GSNO-treated group, hearts were perfused with GSNO (200 μmol/l) for 5 min before freeze-clamping. In the ischemia protocol, hearts were arrested after 20 min with a 3 min infusion of St. Thomas' II cardioplegic solution at 37°C and subjected to normothermic global ischemia for 10 or 30 min. GSNO (20 or 200 μmol/l) and/or nitronyl nitroxide (400 μmol/l) were added to the cardioplegic solution immediately before use. After the ischemic period, hearts were freeze-clamped and processed for ESR spectroscopy. In separate experiments, hearts were reperfused after 30 min cardioplegic ischemia with oxygenated KHB (which did not contain GSNO) for 2, 20, or 30 min and then freeze-clamped for ESR measurements.

3. Results

3.1. Direct detection of nitrosylmyoglobin formation during cardioplegic ischemia

The ESR spectra of control aerobically-perfused and GSNO (200 μmol/l)-perfused heart samples are shown in Fig. 2A. The spectra reveal the presence of a ubisemiquinone radical (g = 2.0045, ΔH_{pp} = 8.0 ± 0.4 G) and a reduced iron-sulfur center (g = 1.94), presumably associated with the mitochondrial NADH or succinate dehydrogenase [17,18]. Perfusion with GSNO did not alter the ESR spectral pattern (Fig. 2A). The ESR spectrum of 10 min ischemic control heart samples contained a secondary ubisemiquinone or flavine semiquinone radical (g = 2.0045, ΔH_{pp} = 10 ± 0.4 G) along with the reduced iron-sulfur center (Fig. 2B). In contrast, the spectrum obtained from 10 min ischemic GSNO-treated heart samples showed a broad spectrum with anisotropic g-values characteristic of nitrosylmyoglobin (MbNO) [19]. A broad absorption peak at g = 2.08 is usually observed for MbNO in biological systems. The intensity of this signal (marked □) increased with the duration of ischemia (Fig. 2C). Fig. 2D shows the ESR spectrum of the sample (i.e. Fig. 2C) at 15 K. The signal-to-noise ratio in Fig. 2D was higher as a result of increased Boltzmann factor. Furthermore, the intensity of the ubisemiquinone radical was greatly reduced due to power saturation of the radical at 15 K.

The ESR spectrum of heart tissue homogenate at 77 K is

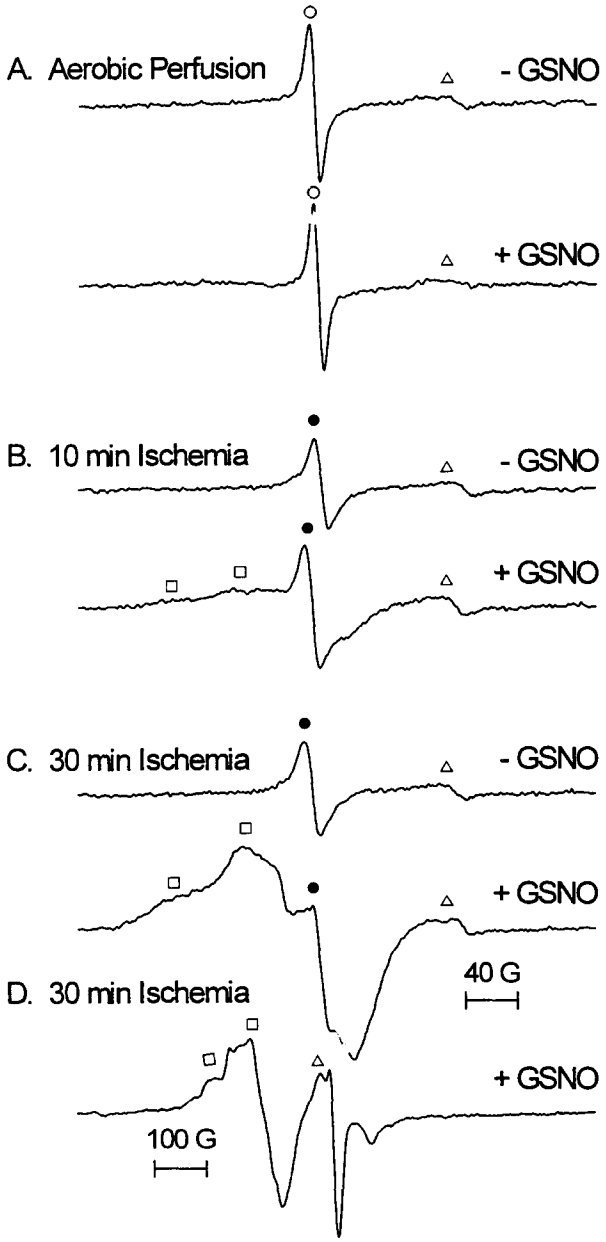

Fig. 2. ESR spectra of (A) control, aerobically perfused (± GSNO), (B) 10 min ischemic (± GSNO), (C) 30 min ischemic (± GSNO) rat heart freeze-clamped and chopped at liquid nitrogen temperature prior to recording of spectra at 77 K, and (D) same as (C) but spectrum recorded at 15 K. Concentration of GSNO was 200 μmol/l. Symbols: ○ = semiquinone radical; ● = secondary semiquinone; △ = reduced iron-sulfur center; and □ = MbNO. Spectrometer conditions used to obtain (A), (B), and (C) are: microwave power, 0.2 mW; modulation amplitude, 3.2 G; gain, 6.3×10^4 scan range, 100 G; scan time, 1 min; time constant, 0.064 s; microwave frequency, 9.2 GHz. Spectrometer conditions used to obtain (D) are: microwave power, 20 mW; modulation amplitude, 5 G; gain, 2.5×10^4; scan range, 1000 G; scan time, 4 min; time constant, 0.128 s; microwave frequency, 9.2 GHz.

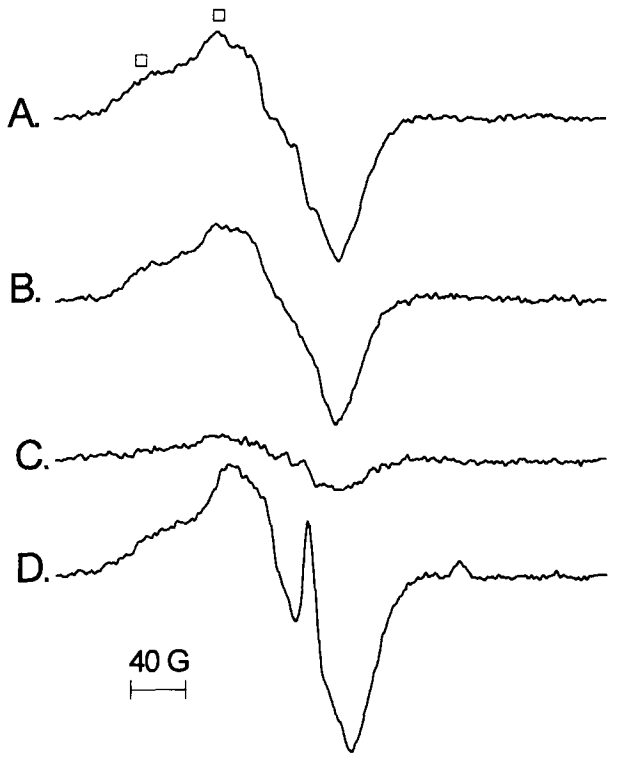

Fig. 3. ESR spectra of (A) homogenized tissue extract of ischemic heart (cf. Fig. 2C) at liquid nitrogen temperature, (B) authentic MbNO adduct prepared from mixing a 100 μM solution of metmyoglobin with 100 μM Angeli's salt, and (C) and (D) difference spectra obtained from subtracting control (−GSNO) spectrum from (+20 μM GSNO) and (+200 μM GSNO) spectrum, respectively. The spike in (C) and (D) represents the residual semiquinone signal. □ indicates line position due to MbNO. Spectrometer conditions for (A) and (B): microwave power, 5 mW; modulation amplitude, 3.2 G; gain, 6.3×10^4; scan range, 100 G; scan time, 1 min; time constant, 0.064 s; microwave frequency, 9.2 GHz.

shown in Fig. 3A. Homogenization of the heart tissue at 0–2°C, apparently, caused the decay of ubisemiquinone and reduced iron–sulfur center signals. The authentic MbNO sample exhibits an ESR spectrum (Fig. 3B) that closely matches the tissue spectrum. Figures 3C and D show the difference spectra obtained from subtracting the control spectrum from 20 and 200 μM GSNO-treated samples. Based on these ESR results, we conclude that myoglobin is an important endogenous intracellular target for •NO produced from •NO donors in myocardial tissue. To prove that MbNO is actually formed from trapping of •NO, we investigated the effect of an exogenous •NO scavenger, nitronyl nitroxide [6,7]. Inclusion of nitronyl nitroxide inhibited the formation of MbNO in GSNO-treated hearts (data not shown).

3.2. Effect of aerobic reperfusion on MbNO signal

Fig. 4A–C show ESR spectra of aerobically-reperfused hearts following 30 min of cardioplegic ischemia. As can be seen, control and GSNO-treated hearts (Fig. 4A) reperfused for 2 min exhibit an increase in the intensity of ubisemiquinone radical signal. With increasing reperfusion time, there was a decrease in the signal intensity due to both ubisemiquinone and MbNO (Fig. 4B and C). Of interest is the finding that even after 30 min of aerobic reperfusion, a significant amount of MbNO was still present in the myocardial tissue. Furthermore, the presence of GSNO in cardioplegia did not affect the formation of the ubisemiquinone radical (Fig. 4A).

4. Discussion

The present study demonstrates that formation of MbNO can be used as an endogenous intracellular marker of •NO released from •NO donors in myocardial tissue. Upon aerobic reperfusion, MbNO remains persistent in myocardial tissues, but decays gradually with time. Formation of ubisemiquinone free radical in reperfused myocardial tissue treated with GSNO was unaffected.

4.1. Mechanism of MbNO formation during ischemia

Molecular oxygen binds reversibly to the reduced form of myoglobin (Mb^{II}) to produce oxymyoglobin (Mb^{II}-O_2) establishing the following equilibrium: ($Mb^{II} + O_2 \rightleftharpoons Mb^{II}O_2$). During aerobic perfusion, the most predominant species in myocytes is oxymyoglobin. During global ischemia, deoxymyo-

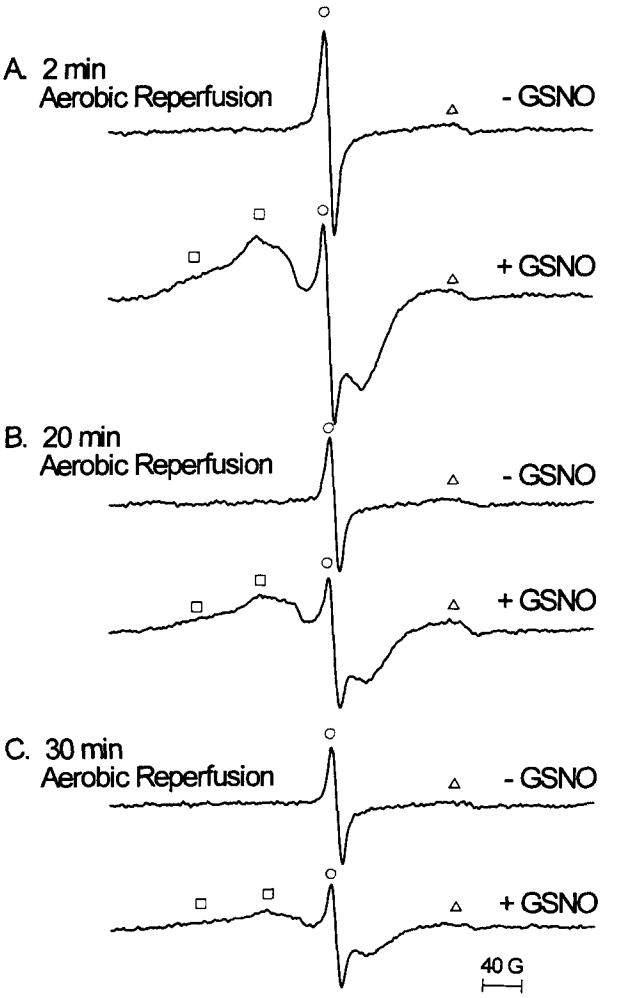

Fig. 4. ESR spectra of freeze-clamped and chopped rat hearts aerobically reperfused for 2, 20, or 30 min after 30 min ischemia with or without GSNO (200 μmol/l). Symbols: ○ = semiquinone radical; △ = reduced iron-sulfur center; and □ = MbNO. Spectrometer conditions: microwave power, 0.2 mW; modulation amplitude, 3.2 G; gain, 6.3×10^4; scan range, 100 G; scan time, 1 min; time constant, 0.064 s; microwave frequency, 9.2 GHz.

globin (Mb^{II}) accumulates. •NO reacts rapidly with deoxymyoglobin to form nitrosylmyoglobin (MbNO); (Mb^{II} + •NO ⇌ MbNO). With increasing ischemic duration, the concentration of MbNO in myocardial tissues increases (cf. Fig. 2B and C). During aerobic reperfusion, molecular oxygen slowly displaces •NO from MbNO (i.e. MbNO + O_2 ⇌ Mb^{III} + NO_3^-). This reaction probably accounts for the slow disappearance of the ESR signal during aerobic reperfusion. The exact mechanism of displacement of •NO by O_2 is not clear, however, this reaction may account for the formation of nitrate from •NO in myocardial tissue and effluents during ischemia and reperfusion.

4.2. Implications in cardioprotection and cardiac transplantation

Arduini et al. [10] have shown that ferrylmyoglobin is formed in isolated ischemic heart. They have postulated that ferrylmyoglobin is formed from the reaction between deoxymyoglobin and hydrogen peroxide produced during ischemia. The potent oxidizing ability of ferrylmyoglobin has been the suggested cause of oxidative damage during ischemia and reperfusion. In contrast to deoxymyoglobin, MbNO does not react with H_2O_2 to form ferrylmyoglobin. In addition, •NO reportedly inhibits H_2O_2/myoglobin-induced lipid oxidation [20,21]. In other studies, the reaction between protein radical associated with ferrylmyoglobin and •NO has been proposed to account for the suppression of the peroxidative damage induced by myoglobin and hydroperoxides [20–22].

ESR spectroscopy has provided direct evidence for increased formation of •NO during rejection of rat heart allograft. ESR spectra of iron-nitrosyl, non-heme nitrosyl, and nitrosylmyoglobin were detected in the blood and at the site of allograft rejection [23]. Pretreatment with immunosuppressive agents greatly decreased the ESR signal. Thus, ESR could be used as a sensitive assay to monitor intracellular •NO formation in organ transplantation. Recently, it has been shown that cardiac preservation can be enhanced in a heterotopic rat model by supplementation of heart storage solutions with •NO donors [24]. Formation of MbNO during cardiac preservation with •NO donors is a distinct possibility.

The slow displacement of •NO from MbNO by oxygen during early reperfusion implies that myoglobin is unable to facilitate intracellular oxygen transport to mitochondria. This has been reported to reduce the rate of mitochondrial respiration and to elevate the NADH/NAD^+ ratio [25,26]. Recently, it has been shown that GSNO inhibits oxygen consumption by isolated rat skeletal muscle mitochondria [27]. Reversible inhibition of respiration during early reperfusion can contribute to the protection of reperfused hearts, since inhibitors of mitochondrial respiration (cyanide and amytal) have been shown to reduce mitochondrial Ca^{2+} accumulation and intracellular enzyme leakage in reoxygenated myocardium [28,29]. It has been hypothesized recently that •NO may be an important physiological regulator of mitochondrial respiration [30]. Also, as mentioned earlier, ferrylmyoglobin formation in reperfused myocardial tissues, following ischemia, has been suggested as the cause of cardiac dysfunction [10,31]. It is likely that •NO donors could inhibit formation of this oxidant, thus protecting against the oxidative tissue injury.

In conclusion, we have shown in this study that ESR monitoring of MbNO can be used as an intracellular marker of •NO release in muscle tissues. Myoglobin appears to be an effective intracellular trap for nitric oxide. Formation of MbNO may contribute to the protective effects of •NO donors in ischemic/reperfused hearts.

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