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Original article

Cardioprotection and mitochondrial *S*-nitrosation: Effects of *S*-nitroso-2-mercaptopropionyl glycine (SNO-MPG) in cardiac ischemia–reperfusion injury

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Abstract

Mitochondrial dysfunction is a key pathologic event in cardiac ischemia–reperfusion (IR) injury, and protection of mitochondrial function is a potential mechanism underlying ischemic preconditioning (IPC). Acknowledging the role of nitric oxide ($NO^{\bullet}$) in IPC, it was hypothesized that mitochondrial protein *S*-nitrosation may be a cardioprotective mechanism. The reagent *S*-nitroso-2-mercaptopropionyl-glycine (SNO-MPG) was therefore developed to enhance mitochondrial *S*-nitrosation and elicit cardioprotection. Within cardiomyocytes, mitochondrial proteins were effectively *S*-nitrosated by SNO-MPG. Consistent with the recent discovery of mitochondrial complex I as an *S*-nitrosation target, SNO-MPG inhibited complex I activity and cardiomyocyte respiration. The latter effect was insensitive to the $NO^{\bullet}$ scavenger c-PTIO, indicating no role for $NO^{\bullet}$-mediated complex IV inhibition. A cardioprotective role for reversible complex I inhibition has been proposed, and consistent with this SNO-MPG protected cardiomyocytes from simulated IR injury. Further supporting a cardioprotective role for endogenous mitochondrial *S*-nitrosothiols, patterns of protein *S*-nitrosation were similar in mitochondria isolated from Langendorff perfused hearts subjected to IPC, and mitochondria or cells treated with SNO-MPG. The functional recovery of perfused hearts from IR injury was also improved under conditions which stabilized endogenous *S*-nitrosothiols (i.e. dark), or by pre-ischemic administration of SNO-MPG. Mitochondria isolated from SNO-MPG-treated hearts at the end of ischemia exhibited improved Ca^{2+} handling and lower ROS generation. Overall these data suggest that mitochondrial *S*-nitrosation and complex I inhibition constitute a protective signaling pathway that is amenable to pharmacologic augmentation.
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1. Introduction

Ischemia–reperfusion (IR) injury is detrimental to cardiac energy metabolism and contractility. Much of the cellular damage observed in IR injury is the result of events at the mitochondrial level, such as Ca^{2+} overload and the over production of reactive oxygen species (ROS) [1]. These events lead to opening of the mitochondrial permeability transition (PT) pore, cytochrome *c* release, and subsequent apoptosis or necrosis [2–4]. The extent of cellular damage in IR is dependent on the length of ischemia, and interestingly brief periods of ischemia are known to trigger signaling pathways that protect against longer ischemic insults; a phenomenon known as ischemic preconditioning (IPC) [5]. Despite numerous studies the precise mechanisms of IPC are still under debate, but the preservation of mitochondrial function is believed to be an important end-point in IPC signaling. Several protective IPC mechanisms are intrinsic to mitochondria, including opening of K^{+}_{ATP} channels [6–8], activation of uncoupling proteins (UCPs) [9–11], modulation of ROS generation [12], or regulation of signaling pathways that impact on the PT pore [13,14]. The complex interplay between these mitochondrial IPC end-points and the upstream cytosolic signals that control them is an area of acute interest.

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Nitric oxide ($NO^{\bullet}$) is a signaling molecule soundly implicated in the mechanism of IPC [15–17]. Potential sources of cardioprotective $NO^{\bullet}$ include endogenous production by $NO^{\bullet}$ synthases (NOSs) [15,18], or exogenous administration of various $NO^{\bullet}$ precursors including *S*-nitroso-glutathione (GSNO) [19], nitroglycerine [20], or nitrite [21]. Notably, $NO^{\bullet}$ can control a number of mitochondrial functions such as Ca^{2+} accumulation and PT pore opening [1,22]. However, as with many other IPC signals, the molecular mechanisms by which $NO^{\bullet}$ acts at the mitochondrial level in IPC are poorly understood.

S-nitros(yl)ation is the reversible $NO^{\bullet}$-mediated modification of thiols resulting in generation of *S*-nitrosothiols (SNOs), and is proposed to be a mechanism of $NO^{\bullet}$-mediated cellular regulation [23–26]. Recently, we suggested that mitochondrial *S*-nitrosation is a potential mechanism of $NO^{\bullet}$ signaling in IPC [27], with one particular example being the *S*-nitrosation and reversible inhibition of mitochondrial respiratory complex I [27,28]. Notably, it has been demonstrated that reversible complex I inhibition by amobarbital is cardioprotective [29,30], and this led us to hypothesize that mitochondrial *S*-nitrosation and subsequent reversible inhibition of the respiratory chain could be a therapeutic avenue for cardioprotection.

Low molecular weight SNOs such as GSNO and *S*-nitrosocysteine (SNOC) have previously been used as *S*-nitrosating agents [23,25], and are known to elicit cardioprotection [31], but are pleiotropic in their actions. Therefore, it was hypothesized that administration of a low molecular weight SNO that accumulates preferentially in mitochondria would deliver cardioprotection at low doses. The parent thiol used in this study was 2-mercaptopropionyl glycine (MPG), which has previously been shown by radiolabeling to accumulate in mitochondria [32], and is known to be both mitochondria- and cardio-protective in IR injury [33,34]. MPG was *S*-nitrosated to yield the S-nitrosothiol SNO-MPG (Fig. 1), which is herein shown to *S*-nitrosate mitochondria, inhibit complex I, and protect cardiomyocytes and perfused hearts from IR injury.

2. Materials and methods

2.1. Animals, chemicals, and reagents

Male Sprague–Dawley rats (200–250 g) were from Harlan (Indianapolis, IN) and were maintained under the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. Crude type II collagenase was from Worthington Biochemical (Lakewood, NJ) and was stripped of endotoxin by chromatography on AffinityPak™ Detoxi-Gel™ columns (Pierce, Rockford, IL). All other chemicals were analytical grade from Sigma (St. Louis, MO) unless otherwise stated.

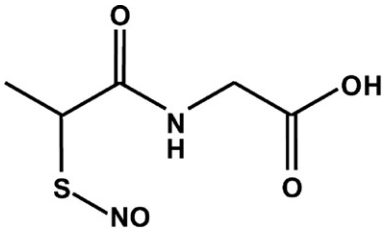

Fig. 1. Chemical structure of SNO-MPG.

2.2. SNO preparation and quantification

Synthesis of GSNO was performed as previously described [27]. Synthesis of SNO-MPG was performed by combining MPG (150 mM) with $NaNO_2$ (150 mM) and HCl (0.5 M). Prior to each experiment, SNO concentration was determined spectrophotometrically at 334 nm ($\varepsilon = 855\ M^{-1}$), and SNO-MPG solutions below 95% purity were discarded.

2.3. Heart perfusions

Isolated rat hearts were retrograde perfused in Langendorff mode under constant flow, essentially as described [10]. Hearts were subjected to one of the following protocols: (i) IR consisting of 25 min ischemia plus 30 min reperfusion; (ii) IR in the dark (laboratory lights switched off); (iii) IR in the dark with SNO-MPG (10 μM) infused into the perfusion cannula just above the aorta for 20 min prior to ischemia; (iv) IPC plus ischemia, consisting of 3 cycles of 5 min occlusion followed by 5 min of reperfusion each, followed by the 25 min global ischemia, without reperfusion; (v) ischemia alone without reperfusion, in the dark; (vi) SNO-MPG plus ischemia alone without reperfusion, in the dark.

2.4. Cardiomyocyte isolation and incubations

Ca^{2+} tolerant adult rat ventricular cardiomyocytes were isolated as described previously [35], except for the use of endotoxin-stripped collagenase (see above). The protocol yielded $\sim 4 \times 10^6$ cells per heart, with ~85% of cells rod-shaped and excluding Trypan blue. Cell count was adjusted to 10^6/ml and cells were kept in Krebs Henseleit (KH) buffer with 2% (w/v) bovine serum albumin (BSA, Fraction V, Fisher Scientific, Pittsburgh PA), in a shaking water bath (80 cycles/min) prior to incubations. Cells were divided into the following treatment groups: (i) normoxia, 95%O_2/5%CO_2 at pH 7.4; (ii) hypoxia-reoxygenation (HR) comprising 1 h of hypoxia (95% N_2/5%CO_2, glucose-free KH buffer, pH 6.5), followed by 30 min of reoxygenation (95%O_2/5%CO_2, glucose-replete KH buffer, pH 7.4); (iii) HR plus IPC, comprising 1×20 min hypoxia plus 20 min reoxygenation, followed by HR as above; (iv) HR plus GSNO, comprising treatment with 10, 20 or 100 μM GSNO for 20 min prior to HR; (v) HR plus SNO-MPG, comprising treatment with 10 or 20 μM SNO-MPG for 20 min prior to HR; (vi) HR plus MPG, comprising treatment with 20 μM MPG for 20 min prior to HR; (vii) HR plus SNO-MPG plus ODQ, comprising treatment with 20 μM SNO-MPG as in (v) above, plus addition of 10 μM of the soluble guanylate cyclase (sGC) inhibitor 1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ), 5 min prior to SNO-MPG addition; (viii) HR plus SNO-MPG plus light, in which cells treated with 20 μM SNO-MPG as in (v) above, were exposed to 2 min illumination by a cold fiber-optic white light source, immediately prior to HR. All incubations utilized 5×10^5 cells in a 5 ml buffer

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