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ScienceDirect
Nitric Oxide 18 (2008) 157–167
NITRIC OXIDE Biology and Chemistry
www.elsevier.com/locate/yniox

Differential role of *S*-nitrosylation and the NO–cGMP–PKG pathway in cardiac contractility

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Received 1 March 2007; revised 5 September 2007
Available online 1 October 2007

Abstract

The role of nitric oxide (NO) in cardiac contractility is complex and controversial. Several NO donors have been reported to cause positive or negative inotropism. NO can bind to guanylate cyclase, increasing cGMP production and activating PKG. NO may also directly *S*-nitrosylate cysteine residues of specific proteins. We used the isolated rat heart preparation to test the hypothesis that the differential inotropic effects depend on the degree of NO production and the signaling recruited. SNAP (*S*-nitroso-*N*-acetylpenicillamine), a NO donor, increased contractility at 0.1, 1 and 10 μM. This effect was independent of phospholamban phosphorylation, was not affected by PKA inhibition with H-89 (*N*-[2((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide), but it was abolished by the radical scavenger Tempol (4-hydroxy-[2,2,4,4]-tetramethyl-piperidine-1-oxyl). However, at 100 μM SNAP reduced contractility, effect reversed to positive inotropism by guanylyl cyclase blockade with ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), and abolished by PKG inhibition with KT5823, but not affected by Tempol. SNAP increased tissue cGMP at 100 μM, but not at lower concentrations. Consistently, a cGMP analog also reduced cardiac contractility. Finally, SNAP at 1 μM increased the level of *S*-nitrosylation of various cardiac proteins, including the ryanodine receptor. This study demonstrates the biphasic role for NO in cardiac contractility in a given preparation; furthermore, the differential effect is clearly ascribed to the signaling pathways involved. We conclude that although NO is highly diffusible, its output determines the fate of the messenger: low NO concentrations activate redox processes (*S*-nitrosylation), increasing contractility; while the cGMP–PKG pathway is activated at high NO concentrations, reducing contractility.

Keywords: Nitric oxide; Nitric oxide synthase; NOS; Rat heart; Langendorff; Tempol; SNAP

Nitric oxide (NO) plays a role in almost all aspects of cardiac function: contractility [1] heart rate [2] and remodeling [3]. Concerning contractility, the effects of exogenous NO appears to be biphasic [4]. In general, when used at high concentrations (near 100 μM of a NO donor) the observed response is a depression of contractility [5–7] but when used at lower concentrations, increases in contractility have been reported [8–11]. A clear cut conclusion cannot be drawn from those reports because different preparations from different species, as well as different NO donors and experimental protocols have been used. Furthermore, the mechanisms underlying NO effects on cardiac contractility still remain controversial and this can be associated with the fact that NO biology is extremely sensitive to factors such as the chemical nature and the kinetics of NO delivery of the donors, and the redox status of the preparation used. In a physiological context, a biphasic behavior for NO could reflect the role that the different nitric oxide synthase (NOS) isoforms (NOS1 and NOS3) play in the regulation of contractility [12]; NOS1 enhancing contractility and NOS3 regulating it negatively. These contrasting effects have been related to the specific subcellular localization of the two NOS isoforms [1].

Classically, the synthesis of NO was related to the activation of the enzyme guanylate cyclase, after binding to its

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doi:10.1016/j.niox.2007.09.086

heme group, leading to an increase in the rate of conversion of GTP to cGMP [13]. Among other features, cGMP is able to activate protein kinase G (PKG). In the heart, targets for PKG include troponin I [14], L-type calcium channel [15] and cyclic nucleotides phosphodiesterases [16] with their respective impact on the contractile status of the myocardium. In addition, *S*-nitrosylation of cysteine residues has emerged as an important feature of NO signaling. Through this post-translational modification, NO is able to regulate the function of enzymes, ion channels and structural proteins [17]. In the heart, *S*-nitrosylation has been suggested as a mechanism by which NO is able to modulate the ryanodine receptor (RyR2) and the L-type calcium channel, two key proteins partaking in excitation-contraction coupling [18].

We postulate that low levels of NO can activate the *S*-nitrosylation pathway, leading to an increase in heart contractility, independently of cGMP; whereas higher levels of NO activate the cGMP–PKG pathway causing a reduction in contractility, indeed overruling the former pathway. To assess this hypothesis, we used different concentrations of SNAP, a *S*-nitrosothiol and a NO donor, in the isolated rat heart preparation, in the absence or presence of different blockers of the cGMP pathway. Consistent with the hypothesis, we show that SNAP can activate *S*-nitrosylation in a redox-sensitive manner at low concentrations, and cGMP synthesis at higher concentrations, with opposite roles concerning cardiac contractility. The increase in *S*-nitrosylation is associated with an increase in contractility and the increase in intracellular cGMP is correlated with a decrease in contractility in the isolated heart through activation of PKG.

Experimental procedures

Animals

Male Sprague–Dawley rats, weighting 290–300 g. were obtained from the Animal Facility of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile. All protocols were approved by the Institutional Bioethics Committee of the Pontificia Universidad Católica de Chile and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

Reagents

8Br-cGMP, H-89 (*N*-[2((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide), isoproterenol hydrochloride (ISO), ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), SNAP (*S*-nitroso-*N*-acetylpenicillamine), and Tempol (4-hydroxy-[2,2,4,4-tetramethyl-piperidine-1-oxyl]), were purchased from Calbiochem (La Jolla, CA). Unless stated otherwise, the rest of the reagents were obtained from Merck (Darmstadt, Germany).

Isolated heart preparation

Rats were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and pre-medicated with 1000 UI heparin i.p. Hearts were rapidly excised and perfused through the aorta with Krebs-Henseleit buffer (equilibrated with a gas mixture of 95% O_2 and 5% CO_2 at 37°), using a peristaltic pump (Gilson Miniplus 3, France). A polyvinyl chloride balloon connected to a pressure transducer by a polyethylene P-50 cannula (Clay Adams-Becton Dickinson, Sparks, MD), was placed through the left atrium and mitral valve into the left ventricle. The balloon was filled with saline to determine isovolumetric intraventricular pressure. Perfusion flow was increased gradually until reaching 10 ml/min and kept constant throughout in order to avoid the Gregg effect [19]. The hearts were placed in a heated chamber and paced at 360 beats/min with platinum electrodes, using a Grass stimulator (pulses of 5 V, 1 ms). Left ventricular pressure (LVP), and coronary perfusion pressure (CPP) were measured continuously with pressure transducers (P23XL, Ohmeda Instruments, Madison, WI, USA), and digitized (Chart, ADI Instruments, New South Wales, Australia), to obtain the rate of change in left ventricular pressure (dP/dt). Minimal diastolic pressure was held constant at 5–10 mm Hg during the experiment.

NO_x measurements

The amount of NO and NO_2^- (NO_x) in SNAP solutions was determined by chemiluminescence as described by Figueroa et al. [20]. SNAP solutions were freshly prepared in gassed Krebs-Henseleit buffer, in identical conditions as those used in the Langendorff preparation. After 30 min, an aliquot of this solution was injected into the reaction chamber of an NO Analyzer (Sievers 280), filled with acetic acid and potassium iodine. In these conditions, nitrites, but not nitrates are reduced to NO, therefore the content of NO plus nitrite is measured.

Phospholamban phosphorylation

In some experiments, the content of phospholamban, both total protein and the form phosphorylated at ser16 (phospho-phospholamban) were assessed by Western blotting of heart homogenates. Briefly, a portion of the left ventricle (~200 mg) was excised at the specified time of the stimulation protocol, and quickly homogenized by an Ultraturrax in 1 mL of cold Tris–HCl buffer (100 mM pH 7.4) containing antiproteases (5 mM EGTA, 1 μg/mL Aprotinine, 1 mM benzamidine, 10 μg/mL leupeptin, 10 μg/mL pepstatin-A, 2 mM PMSF, 200 μg/mL SBTI). The homogenate was centrifuged 30 s at 100*g* and the supernatant was mixed with Laemli's buffer and resolved in 12% SDS–PAGE. Gels were blotted on nitrocellulose and tested sequentially with polyclonal anti phospho Ser16-phospholamban (Upstate, Lake Placid, NY) and monoclonal anti phospholamban antibodies (Abcam Plc, Cambridge, UK). The intensity of the signal was evaluated using the Image J program (NIH public domain software).

S-Nitrosylation assay

Protein *S*-nitrosylation was assessed by using the biotin switch method [21,22], according to the instructions of a commercially available kit (Nitroglo kit, Perkin Elmer, Boston, MA, USA). Briefly, a left ventricular sample (~200 mg) was excised and homogenized, in assay buffer with antiproteases as indicated above. The homogenate was centrifuged 10 min at 750*g* at 4 °C, and in the supernatant, the free sulfhydryls groups were blocked with methyl methanethiosulfonate. Nitrosylated proteins present in the cardiac homogenate were reduced (1.6 mM ascorbic acid) and the newly formed thiols were reacted with biotin-HPDP. After this, proteins were resolved in 5%, 7.5% or 12% SDS–PAGE and the biotinylated proteins were visualized using an anti-biotin antibody (Cell Signaling, Beverly, MA), or HRP–streptavidin (Sigma Chemical. St. Louis, MO). Specific *S*-nitrosylation of cardiac RyR receptor was evaluated in separate assays, in which proteins were resolved in 3–8% Tris–acetate gradient gels and after detection of biotin, the membrane was stripped and reincubated with a monoclonal antibody against RyR2 (Affinity Bioreagents, Golden, CO, USA).

cGMP assay

In one series, immediately at the end of treatment with SNAP, hearts were stopped by immersion in ice-cold TCA 10% (w/v), quickly weighed and homogenized with an Ultraturrax in 3 mL of TCA. After centrifuga-