

The $\alpha 1$ isoform of soluble guanylate cyclase regulates cardiac contractility but is not required for ischemic preconditioning

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Abstract Nitric oxide (NO)-dependent soluble guanylate cyclase (sGC) activation is an important component of cardiac signal transduction pathways, including the cardioprotective signaling cascade induced by ischemic preconditioning (IPC). The sGC α subunit, which binds to the common sGC $\beta 1$ subunit, exists in two different isoforms, sGC $\alpha 1$ and sGC $\alpha 2$, but their relative physiological roles remain unknown. In the present study, we studied Langendorff-perfused isolated hearts of genetically engineered mice lacking functional sGC $\alpha 1$ (sGC $\alpha 1$ KO mice), which is the predominant isoform in the heart. Our results show that the loss of sGC $\alpha 1$ has a positive inotropic and lusitropic effect on basal cardiac function, indicating an important role for sGC $\alpha 1$ in regulating basal myocardial contractility. Surprisingly, IPC led to a similar 35–40% reduction in infarct size and concomitant protein kinase C ϵ (PKC ϵ) phosphorylation in both wild-type (WT) and sGC $\alpha 1$ KO hearts subjected to 40 min of global ischemia and reperfusion. Inhibition of the activation of all sGC isoforms

by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μ mol/L) completely abolished the protection by IPC in WT and sGC $\alpha 1$ KO hearts. NO-stimulated cGMP production was severely attenuated in sGC $\alpha 1$ KO hearts compared to WT hearts, indicating that the sGC $\alpha 2$ isoform only produces minute amounts of cGMP after NO stimulation. Taken together, our results indicate that although sGC $\alpha 1$ importantly regulates cardiac contractility, it is not required for cardioprotection by IPC. Instead, our results suggest that possibly only minimal sGC activity, which in sGC $\alpha 1$ KO hearts is provided by the sGC $\alpha 2$ isoform, is sufficient to transduce the cardioprotective signal induced by IPC via phosphorylation of PKC ϵ .

Keywords Soluble guanylate cyclase · Ischemic preconditioning · Isolated heart · Contractility · Nitric oxide

Introduction

Cardiac ischemia is the leading cause of death in the world. The preferred treatment is the rapid restoration of blood flow to the myocardium, but reperfusion of the tissue is in itself responsible for significant additional tissue damage [59]. A large number of studies have confirmed that ischemic preconditioning (IPC) can significantly reduce infarct size after myocardial ischemia–reperfusion [58]. IPC was shown to have two phases of cardioprotection against ischemic injury: the initial or “early” phase of preconditioning lasting approximately 2–3 h after the IPC stimulus [36], and a second window of protection, or “delayed” preconditioning, occurring 12–24 h after the IPC stimulus and lasting for 2–3 days [32]. A similar cardioprotection is observed after ischemic postconditioning (IPostC) [61].

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Studies examining the role of nitric oxide (NO) in myocardial ischemia and reperfusion have shown that endogenous NO is not only involved in the adaptation to cardiac ischemia [20, 22], but also plays a major role in cardioprotection after both IPC and IPostC [8, 21, 35, 49, 51]. Delayed preconditioning was shown to rely on both endothelial and inducible NO synthase [16, 55], while a recent study confirms that early IPC also requires endothelial NO synthase [53]. The precise mechanisms of cardioprotection, however, remain incompletely understood. Elucidation of the mechanisms responsible for cardioprotection induced by IPC and IPostC might lead to the development of new therapeutic strategies to improve the outcome of patients with ischemic heart disease [19, 40].

One of the most important cardiovascular receptors for NO is soluble guanylate cyclase (sGC) [39, 47]. sGC has been shown to be required for the infarct size reduction afforded by IPostC [43, 57], and it has been suggested that a crucial step in the trigger phase of cardioprotective IPC signaling is the stimulation of sGC activity [9, 45]. sGC-derived cGMP is believed to exert its cytoprotective effects by activating cGMP-dependent protein kinase (PKG) [15], which then directly or indirectly phosphorylates the ϵ isoform of protein kinase C (PKC ϵ) [10], leading to the opening of mitochondrial ATP-sensitive K⁺-channels (mitoKATP) and subsequent reactive oxygen species production [2, 18, 38, 44]. This ultimately leads to inhibition of the mitochondrial permeability transition at reperfusion, which protects mitochondria from rupturing and prevents cardiomyocyte death [11, 35].

In addition, some of the effects of NO on the regulation of cardiac contractility have also been proposed to be mediated by sGC [27]. It has been suggested that sGC, via a PKG-dependent mechanism, can inhibit the influx of Ca²⁺ into cardiomyocytes [1] and attenuate the myofilament sensitivity to Ca²⁺ [30]. cGMP can also stimulate phosphodiesterase 2 activity, which counteracts cAMP generation and in this way blunts positive inotropic signaling [34].

sGC is a heterodimeric enzyme, consisting of an α and a β subunit, which exist in different isoforms. sGC α 1 and sGC α 2, bound to the common β 1 subunit, have been characterized as the catalytically active forms. The sGC α 1 subunit has been shown to be the most abundant isoform in most tissues, including the heart [3, 33]. However, the relative physiological and pathophysiological importance of sGC α 1 versus sGC α 2 signaling has not yet been revealed. We have previously described the generation of genetically modified mice deficient in functional sGC α 1 (sGC α 1KO mice) [5, 6] and found that male sGC α 1KO mice on a 129/S6 genetic background are hypertensive, although this phenotype is lost after backcrossing the mutation to the C57BL/6J background. Interestingly,

129/S6 sGC α 1KO mice also display an enhanced cardiac contractility phenotype.

In this study, we used both sGC α 1KO mice as well as total pharmacological sGC inhibition to elucidate the role of sGC and its isoforms in the signal transduction mechanisms responsible for the early phase of cardioprotection afforded by IPC.

Materials and methods

Mice

All animal procedures were performed in accordance with the guidelines published in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Mice lacking a functional sGC α 1 subunit (sGC α 1KO mice) were generated previously [6] and were backcrossed for eight generations on the C57BL/6J genetic background. Wild-type (WT) mice on the C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME). Male mice, matched for age (range 2–4 months) and body weight (range 23–30 g), were used throughout the study.

Langendorff preparation

Mice were administered 200 U heparin by intraperitoneal injection and anesthetized with 50 mg/kg pentobarbital. The heart was excised and quickly transferred to ice-cold perfusion buffer (a modified Krebs-Henseleit buffer, containing, in mmol/L: NaCl 118.5; NaHCO₃ 25; glucose 11; KCl 4; MgSO₄ 1.2; KH₂PO₄ 1.2; pyruvate 1; CaCl₂ 1.8; gassed and equilibrated with 95% O₂ and 5% CO₂ at 37°C). The aorta was cannulated immediately, and the heart was mounted on the Langendorff apparatus and perfused at a constant pressure of 70 mmHg. Hearts were paced at 7 Hz. The left ventricular (LV) diastolic pressure was initially set at 5–10 mmHg using a fluid-filled balloon inserted into the LV, which also contained the tip of a Millar SPR-671 pressure transducer (ADInstruments, Colorado Springs, CO). Coronary flow rate was measured using an N1 in-line flow probe and a T106 flow meter (Transonic Systems, Ithaca, NY). Coronary flow rate and LV pressure were constantly measured, and heart rate, LV developed pressure (LVDevP), and maximum and minimum rate of LV pressure change (dP/dt_{max} and dP/dt_{min}, respectively) were calculated from the LV pressure signal using a Powerlab 8/30 data acquisition system and Chart Pro software (ADInstruments, Colorado Springs, CO).

Experimental protocols

After a stabilization period of approximately 20 min perfusion on the Langendorff system, hearts were subjected to a preconditioning protocol of four cycles of 5 min each of global ischemia followed by 5 min of reperfusion. Hearts were then subjected to 40 min of global ischemia, followed by 60 min of reperfusion. Hearts were then removed from the Langendorff apparatus and their infarct size was determined as described below. Control hearts were subjected to the 40-min global ischemia and subsequent reperfusion without preconditioning. In a separate group of experiments, hearts were frozen in liquid nitrogen after 20 min of reperfusion after the prolonged ischemia for immunoblot analysis. For a subgroup of hearts, the perfusion buffer was supplemented with 1H-[1,2,4]oxadiazolo [4,3-a]quinoxaline-1-one (ODQ, Cayman Chemical, Ann Arbor, MI) at a final concentration of 10 $\mu\text{mol/L}$, a dose that was previously shown to inhibit all sGC activation [23, 46].

In a separate set of experiments, after stabilization on the Langendorff setup, the NO donor compound diethylammonium (Z)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO, Cayman Chemical, Ann Arbor, MI) or its vehicle was infused into the perfusion buffer. Hearts were frozen in liquid nitrogen immediately after 90 s of infusion of DEA/NO (100 $\mu\text{mol/L}$) or vehicle.

Infarct size measurements

At the end of reperfusion, hearts were removed from the Langendorff apparatus, and 1 mm cardiac slices were stained with 2,3,5-triphenyltetrazolium chloride (1% wt/vol) as described previously [37]. Computer-assisted planimetry (NIH ImageJ 1.43 h) was used to quantify the myocardial infarction size as a percentage of total myocardial area.

Western blot

Cardiomyocytes were obtained from adult mouse hearts as described previously [24]. Using a Dounce tissue grinder, the isolated cardiomyocytes were then homogenized in buffer containing, in mmol/L: sucrose 250, EDTA 0.1, Tris-HCl (pH 7.4) 50, dithiothreitol 1, and protease inhibitors. Frozen whole hearts were homogenized in buffer containing, in mmol/L: sucrose 250, EDTA 1, Tris-HCl (pH 7.4) 10, and phosphatase and protease inhibitors. After clearing the homogenates by ultracentrifugation at 100,000g, protein concentration in the supernatant was measured with the BCA protein assay (Thermo Scientific, Rockford, IL). Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to

PVDF membranes. The blots were then probed with primary antibodies against sGC α 1 (1:3,000; Abcam, Cambridge, MA), sGC α 2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), phosphoPKC ϵ (Ser729) (1:5,000; Upstate, Lake Placid, NY), total PKC ϵ (1:10,000; Millipore, Temecula, CA), and β -tubulin (1:20,000; Cell Signaling Technology, Danvers, MA). To assess the specificity of the sGC α 2 antibody, a parallel experiment was performed where the antibody was first preincubated for 1 h at room temperature with a fivefold excess of the blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA). Next, the membranes were incubated with the appropriate secondary antibody coupled to HRP (for sGC α 2: anti-goat, 1:100,000; Santa Cruz Biotechnology, Santa Cruz, CA; for all others: anti-rabbit, 1:50,000; Cell Signaling Technology, Danvers, MA), developed with the ECL Advance chemiluminescence kit (GE Healthcare, Piscataway, NJ) and exposed to X-ray film. The film was then scanned and densitometric analysis was performed using NIH ImageJ 1.43 h. Experiments were repeated at least twice for each sample. Phospho-PKC ϵ levels are presented as the ratio of the level of the phosphorylated protein to total protein, normalized to the respective values of control samples without IPC.

cGMP measurements

Frozen cardiac tissue was homogenized in 1 mL ice-cold 10% trichloroacetic acid. The protein pellet obtained after centrifugation was saved to measure original protein concentration in the sample using the BCA protein assay. The supernatants were extracted with water-saturated ether and dried by vacuum centrifugation. After resuspending the samples in assay buffer, cGMP concentration was determined using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations. The obtained results were normalized to the protein concentration in the original sample.

Statistics

All data are presented as mean \pm SEM. Statistical differences among groups were evaluated using the appropriate test as indicated in the text. A value of $P < 0.05$ was considered as significant.

Results

Both sGC α isoforms are expressed in cardiomyocytes

Western blot analysis of homogenates of perfused whole hearts and isolated cardiomyocytes demonstrated the

expression of both the sGC α 1 and sGC α 2 isoform in WT samples (Fig. 1). In samples from sGC α 1KO mice, the sGC α 1-specific band was absent, while sGC α 2 was still detectable. Densitometric analysis of sGC α 2 levels showed that there were no significant differences between WT and sGC α 1KO samples.

sGC α 1 deficiency enhances LV contractility at baseline

Measurements of the functional parameters of Langendorff-perfused hearts, after stabilization and before ischemia or other experimental interventions, indicated an altered basal cardiac function in sGC α 1KO hearts as compared to WT hearts. Both LVDevP and dP/dt_{\max} were higher in isolated sGC α 1KO hearts than in WT hearts

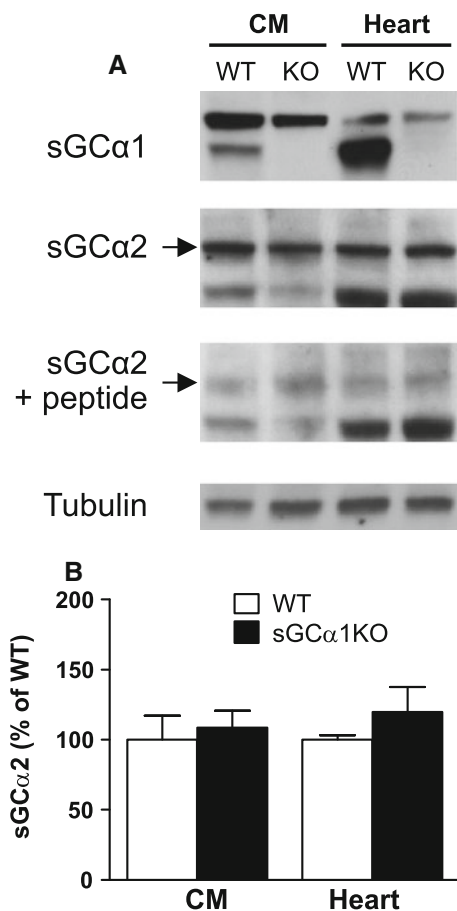


Fig. 1 Immunoblot analysis of the sGC α 1 and sGC α 2 isoforms, as well as tubulin in whole hearts and cardiomyocytes of WT and sGC α 1KO mice. **a** Representative immunoblots of sGC isoforms in homogenates of isolated cardiomyocytes (CM) and perfused whole hearts (heart). The sGC α 2-immunoreactive band was blocked by the corresponding immunization peptide, confirming the specificity of the immunostaining. **b** Quantitative analysis of sGC α 2 expression levels in WT ($N = 4$) and sGC α 1KO ($N = 5$) samples, normalized to the respective WT samples. No significant differences were found between groups

(Fig. 2a, b), demonstrating an increase in contractile activity and inotropy in hearts lacking functional sGC α 1. Pharmacological inhibition of sGC also significantly increased inotropy as evidenced by elevated LVDevP and dP/dt_{\max} in hearts perfused with 10 μ mol/L ODQ. In addition, both the genetic ablation of sGC α 1 and sGC inhibition had a positive lusitropic effect, as measured by a more negative dP/dt_{\min} (Fig. 2c). However, coronary flow rate was similar in all groups, demonstrating that the changes in contractility are not secondary to an increase in substrate or oxygen availability to the heart (Fig. 2d).

Ischemia–reperfusion and cardioprotection by IPC has similar effects in isolated hearts from both wild-type and sGC α 1KO mice

Infarct sizes measured after 40 min of global ischemia and 1 h of reperfusion were comparable between both genotypes ($41 \pm 4\%$ in WT and $40 \pm 2\%$ in sGC α 1KO). Interestingly, IPC markedly reduced infarct size to a similar extent in both genotypes ($26 \pm 2\%$ in WT and $24 \pm 2\%$ in sGC α 1KO; for both: $P < 0.01$ versus control without IPC, by Bonferroni's post hoc test after two-way ANOVA) (Fig. 3). Although IPC improved contractile function after 40 min of global ischemia and 20 min of reperfusion, there was no significant difference in

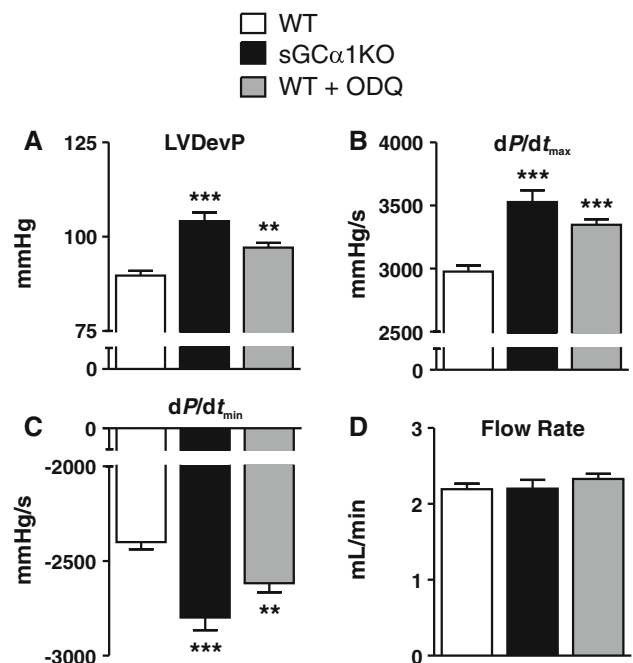


Fig. 2 Basal functional parameters in isolated hearts from male WT ($N = 49$), sGC α 1KO ($N = 32$), and WT mice treated with 10 μ mol/L ODQ ($N = 40$). **a** LV developed pressure (LVDevP); **b** maximum and **c** minimum rate of LV pressure change (dP/dt_{\max} and dP/dt_{\min} , respectively); **d** coronary flow rate. ** $P < 0.01$ and *** $P < 0.001$ versus WT by Bonferroni's post hoc test after one-way ANOVA

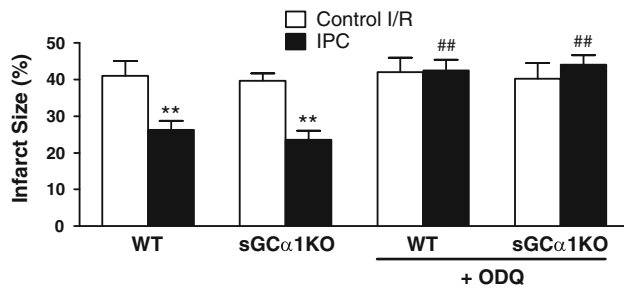


Fig. 3 Infarct size measured after 40 min of global ischemia and 1 h of reperfusion with (IPC) or without (Control I/R) ischemic preconditioning. $N = 6-8$ per group; $**P < 0.01$ versus control I/R without IPC and $###P < 0.01$ versus respective group without ODQ by Bonferroni's post hoc test after two-way ANOVA

functional parameters within or between genotypes (Supplementary Table 1).

Total sGC inhibition abolishes the cardioprotective effect of IPC

In a subgroup of hearts, ODQ (10 $\mu\text{mol/L}$) was added to the perfusion buffer to inhibit the activation of all sGC isoforms [23, 46] (Fig. 3). Although ODQ administration did not have an effect on the infarct size of control WT hearts without IPC, it completely abolished the ability of IPC to reduce infarct size in WT hearts (WT control + ODQ: $42 \pm 4\%$; WT IPC + ODQ: $42 \pm 3\%$). ODQ administration had a similar effect on sGC α 1KO hearts, leading to inhibition of IPC-induced cardioprotection in these hearts as well (infarct sizes: sGC α 1KO control + ODQ: $40 \pm 4\%$; sGC α 1KO IPC + ODQ: $44 \pm 3\%$).

Protection by IPC is associated with sGC-dependent PKC ϵ phosphorylation

Serine residue 729 (Ser729) phosphorylation of PKC ϵ was investigated by immunoblot after 40 min of ischemia and 20 min of reperfusion (Fig. 4). Both in WT and sGC α 1KO hearts, the level of PKC ϵ phosphorylation was increased after IPC, as compared to control hearts that underwent ischemia and reperfusion without IPC. However, in WT hearts that were perfused with ODQ, IPC did not increase PKC ϵ Ser729 phosphorylation.

cGMP production after NO stimulation is severely attenuated in sGC α 1KO hearts

To examine the relative contribution of both sGC α isoforms to cardiac cGMP production, we stimulated isolated WT and sGC α 1KO hearts with a high dose (100 $\mu\text{mol/L}$) of the NO donor compound DEA/NO or vehicle and measured the cGMP content in tissue homogenates. In WT

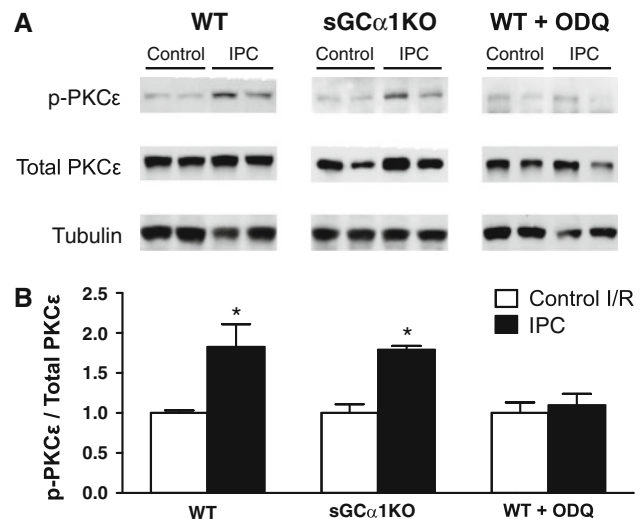


Fig. 4 Western blots showing the phosphorylation of the Ser729 residue on PKC ϵ after IPC in WT and sGC α 1KO hearts. **a** Representative blots are shown for Ser729 phosphorylated PKC ϵ (p-PKC ϵ), total PKC ϵ , and tubulin. Control samples subjected to 40 min ischemia and 20 min of reperfusion without IPC; IPC samples subjected to ischemia and reperfusion with IPC. **b** Graph showing the average ratio of the relative intensity of Ser729 phosphorylated PKC ϵ to total PKC ϵ ($N = 4-6$ samples per group). $*P < 0.05$ versus respective control by unpaired t test

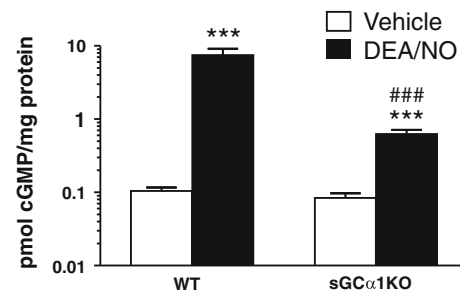


Fig. 5 cGMP concentration in homogenates of WT and sGC α 1KO isolated hearts perfused with buffer containing vehicle or DEA/NO (100 $\mu\text{mol/L}$). $***P < 0.001$ versus vehicle and $###P < 0.001$ versus WT, both by Bonferroni's post hoc test after two-way ANOVA

hearts, we observed a 72-fold increase in cGMP concentration in NO-stimulated samples versus control samples, while we only found a sevenfold increase in sGC α 1KO hearts (Fig. 5). Similarly, we found a significant increase in cGMP secretion in the effluent of WT hearts stimulated with 1 $\mu\text{mol/L}$ DEA/NO, while we found no significant NO-dependent increases in sGC α 1KO hearts or hearts treated with ODQ (Supplementary Fig. 1).

Discussion

The data presented here describe the phenotype of isolated sGC α 1KO hearts perfused in the Langendorff mode and

subjected to ischemia and reperfusion with or without IPC. Firstly, we confirmed the presence of both the sGC α 1 and sGC α 2 isoform in both whole hearts and isolated cardiomyocytes, and demonstrated the absence of the former while the latter remained unchanged in sGC α 1KO tissue. We found that sGC α 1KO hearts exhibited a higher basal contractility than WT hearts, but had a similar infarct size after ischemia and reperfusion as WT hearts. Moreover, IPC had a similar protective effect in both WT and sGC α 1KO hearts, as demonstrated by a comparable reduction of infarct size after ischemia and reperfusion, whereas inhibition of all sGC isoforms abolished cardioprotection afforded by IPC. The IPC-induced cardioprotection was shown to correlate with phosphorylation of PKC ϵ at the Ser729 residue. Finally, we demonstrated that sGC α 1 deficiency severely attenuated the NO-induced cGMP production in the heart. These observations underscore the important role of the sGC α 1 isoform in the regulation of basal contractility. Our results also confirm its role as the predominant cardiac isoform responsible for the bulk of cGMP production, while surprisingly demonstrating that it is not required for cardioprotection. Instead, our results suggest that in sGC α 1KO hearts, the sGC α 2 isoform, which only produces low amounts of cGMP after NO stimulation, is sufficient to transduce the IPC-induced cardioprotective signal to PKC ϵ .

To the best of our knowledge, this is the first study to examine contractile function and IPC in hearts of sGC α 1KO mice using the Langendorff setup. We observed that sGC α 1 deficiency led to enhanced inotropy (higher LVDevP and dP/dt_{\max}) and lusitropy (more negative dP/dt_{\min}) in the heart, an effect that was at least partly mimicked by acute pharmacological inhibition of sGC by ODQ. Of note, we previously observed an increased cardiac contractile phenotype *in vivo* as well in sGC α 1KO mice, albeit on the 129/S6 genetic background [6]. This increased cardiac contractility was, however, absent in sGC α 1KO mice on a C57BL/6J background [5]. Nonetheless, the results presented here suggest that the higher contractility in sGC α 1KO mice at baseline is caused by an inherent cardiac phenotype, which might be masked in the sGC α 1KO mice on the C57BL/6J background *in vivo* due to the influence of circulating neurohumoral factors. Furthermore, our data are in line with other studies suggesting a negative inotropic effect of sGC-derived cGMP in the myocardium [42, 54]. The absence of an effect of ODQ on basal coronary flow, as previously observed in another study using a similar isolated heart perfusion setup [14], most likely reflects the negligible contribution of basal vascular NO-mediated sGC activation in this model.

Several mechanisms have been proposed to be involved in the effects of cGMP on cardiac contractility. One possibility is the PKG-dependent phosphorylation of the

L-type Ca²⁺ channel [48, 56], leading to decreased Ca²⁺ influx into the cardiomyocyte. PKG activation can also increase the phosphorylation of troponin I, thereby decreasing the myofilament sensitivity to Ca²⁺ [30, 50], although this does not explain the lusitropic effect we observed. Alternatively, the PKG-independent activation of cAMP-catabolizing phosphodiesterase might be responsible for the negative inotropic and lusitropic effects of cGMP [60]. Decreased activation of this phosphodiesterase in sGC α 1KO hearts may increase cAMP-dependent protein kinase (PKA) activity, leading to the phosphorylation of phospholamban and troponin I [31], which would increase both inotropy and lusitropy. This effect of PKA is supported by a recent study showing that disruption of PKA localization and activity leads to a decrease in inotropy and lusitropy in isolated perfused hearts [41].

Importantly, our data demonstrate that the α 1 isoform of sGC is not required for the infarct size reduction afforded by IPC in the heart. However, administration of ODQ, which has previously been shown to inhibit both sGC isoforms [46], to the perfusion buffer, abolished the cardioprotective effects of IPC. This finding underscores the key role of sGC in IPC signaling, and is consistent with previous studies indicating a role for sGC in cardioprotection [17, 43, 57]. Furthermore, the observation that ODQ inhibits IPC-induced cardioprotection in sGC α 1KO hearts also suggests that the sGC α 2 isoform is responsible for mediating the cardioprotective signal transduction pathways initiated by IPC in sGC α 1KO hearts.

Moreover, our finding that ODQ has similar effects on cardioprotection in WT and sGC α 1KO hearts demonstrates that there is no compensatory upregulation of alternative, non-sGC-dependent cardioprotective pathways in the sGC α 1KO hearts. Nevertheless, our data do not contradict other studies that indicate a cardioprotective role for non-sGC-dependent effects of NO (e.g., S-nitrosylation [51]) or non-sGC-derived cGMP (e.g., B-type natriuretic peptide signaling [4]). It is likely that these alternative pathways play a role in another phase of cardioprotective signaling. Indeed, Cohen et al. [9] recently proposed that an sGC-independent signaling step is activated in the mediator phase of cardioprotection, while sGC is suggested to play a role in the trigger phase of IPC, and another group observed that natriuretic peptide-induced cardioprotection still requires sGC activation [12].

In this study, we also show that phosphorylation of the Ser729 residue of PKC ϵ is correlated with cardioprotection by IPC, confirming that this kinase is an essential component of the IPC signaling cascade downstream of sGC [10]. Our results imply that cGMP produced by sGC α 2 in sGC α 1KO hearts is sufficient to activate downstream mechanisms that increase PKC ϵ phosphorylation, leading to opening of mitoKATP and other subsequent signaling

pathways that confer protection from cardiac ischemia–reperfusion injury [8, 11, 38, 45]. In WT hearts, the cGMP that mediates the cardioprotective signal might be derived from either sGC α 1 or sGC α 2.

Interestingly, administration of a high dose of an NO donor stimulated cGMP production to a significantly higher level in WT hearts than in sGC α 1KO hearts (approximately, 70-fold vs. 7-fold increase as compared to vehicle control, respectively). Similarly, a lower dose of the NO donor significantly increased cGMP release from WT hearts, but not from sGC α 1KO hearts. These results demonstrate that sGC α 1 is the predominant isoform in the heart responsible for the bulk of cGMP production, and that sGC α 2 activity is still detectable in sGC α 1KO hearts, albeit after administration of a high dose of exogenous NO. These observations are consistent with our previously published results showing that left ventricular extracts from sGC α 1KO mice display a severely attenuated NO-induced increase in sGC activity [6]. Nevertheless, inhibition of all sGC isoforms by ODQ, but not loss of the sGC α 1 subunit, impaired the cardioprotection by IPC, suggesting that the sGC α 2 isoform can be of functional importance despite producing very low amounts of cGMP.

In the current study, we did not detect upregulation of the sGC α 2 subunit in whole heart or isolated cardiomyocyte samples of sGC α 1KO mice, confirming our observations in other tissue types we examined previously [5, 6]. This supports our hypothesis that only very small amounts of sGC-derived cGMP, as provided by sGC α 2 in the sGC α 1KO hearts, are sufficient to transduce an NO-dependent signal in IPC. It is possible that sGC α 2 has a more restricted subcellular localization than sGC α 1, leading to sGC isoform-specific compartmentation of NO-dependent cGMP signaling. Activation of sGC α 2 (or sGC α 1) in this theoretical compartment might be sufficient to transduce the cardioprotective signal induced by IPC. In support of this hypothesis, localized pools of cGMP with differing biological effects have already been shown to exist in cardiomyocytes [7], explaining why cGMP derived from sGC can have different effects than cGMP produced by particulate guanylate cyclase (e.g., cGMP produced by sGC specifically blunts the cardiac contractile response to β -adrenergic signaling [52], while cGMP derived from particulate guanylate cyclase only modulates the contractile response to angiotensin II [25]). Definitive confirmation of the specific role of sGC α 2 in IPC, however, will require further investigation of genetically modified mice lacking a functional sGC α 2 isoform.

Taken together, our data confirm that cardioprotection afforded by IPC is mediated by sGC. However, our study surprisingly shows that the major cardiac isoform, sGC α 1, is not required for this effect. Instead, our results suggest that the much less prominent sGC α 2 isoform is sufficient to

mediate the IPC-induced cardioprotective signaling cascade in sGC α 1KO mice. Since several recent studies showed the ability of sGC stimulators, which activate both sGC α isoforms [13, 26], to protect the heart from ischemic injury [28, 29], it might be of clinical value to develop sGC α 2-specific stimulators. It is conceivable that inducing only very low levels of sGC activity, such as via specific stimulation of the sGC α 2 isoform, might be sufficient to protect the heart. This might have therapeutical benefits over non-specific stimulation of both sGC isoforms, which can be expected to have negative inotropic and lusitropic effects due to the higher levels of cGMP produced by the sGC α 1 isoform.

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Conflict of interest The authors declare that they have no conflict of interest.

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