Research Paper

Heterogeneous Expression of NO-Activated Soluble Guanylyl Cyclase in Mammalian Heart

Implications for NO- and Redox-Mediated Indirect Versus Direct Regulation of Cardiac Ion Channel Function

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INTRODUCTION

Nitrogen oxides exert significant but diverse regulatory effects on cardiac myocytes. Many of these effects are due to modulation of voltage-sensitive ion channel function. The redox-status of NO-related compounds is a critical factor in determining whether indirect (cGMP-dependent) versus direct (cGMP-independent) effects are dominant. However, molecular mechanisms by which different cardiac myocyte types, and associated different ion channel types expressed within them, could achieve selectivity between NO-related indirect versus direct effects are unclear. We have previously demonstrated heterogeneous expression gradients of Type III NO synthase (eNOS) and sarcolemmal superoxide dismutase (ECSOD) in ferret and human ventricle, with both enzymes being highly expressed in right ventricle and left ventricular subepicardium but markedly reduced in left ventricular subendocardium. In this study we extend this previous analysis by analyzing NO-activated soluble guanylyl cyclase (sGC) expression in the heart (ferret and human). We demonstrate that, at both tissue and single myocyte levels, sGC protein expression is heterogeneous, being high in sinoatrial node, right atrium, right ventricle and left ventricular subepicardium, but markedly reduced in left atrium and left ventricular subendocardium. Thus, there is a significant overlap in expression gradients of sGC, eNOS, and ECSOD among distinct cardiac tissue and myocyte types. These gradients positively correlate with both: (i) experimentally measured basal NO production levels; and (ii) expression gradients of specific voltage-gated ion channels (particularly Kv1 and Kv4 channels). Our results provide the first demonstration in the heart of an expressed coupled multienzymatic system for selective regulation of indirect (sGC-dependent) versus direct (sGC-independent) NO- and redox-related modulation of voltage-gated ion channel function in different myocyte types. Our results also have functional implications for NO•/redox-related modulation of ion channels expressed in other cell types, including neurons, skeletal muscle and smooth muscle.

KEY WORDS

NOS isoforms, Soluble guanylyl cyclase, Kv4 channels, NO, Redox modulation, ERG, Ion channel regulation, L-type Ca channels

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ABSTRACT

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Nitrogen oxides exert significant modulatory effects upon cardiac muscle.1-5 Many of these effects are due to regulation of cardiac myocyte ion channel activity. However, the reported effects of NO-related activity on cardiac muscle function and ion channel activity are quite diverse, and in many cases contradictory. While underlying mechanisms are still unclear, two potential mechanisms for such diversity are as follows:

Differences in redox-status and reactivity profiles of various N-oxides. Based upon its known biochemistry,5-7 free radical NO• would be predicted to exert primarily indirect cyclic guanosine monophosphate (cGMP)-dependent effects through activation of soluble guanylyl cyclase (sGC).6,10 Increased intracellular cGMP levels would then result in subsequent activation/inhibition of various potential sGC-dependent mediated cascade pathways, including pathways involving protein kinase G (PKG) and/or various cGMP-dependent phosphodiesterases (cGMP-PDEs):

Eqn 1. NO• + M (heme group in sGC) → M – NO (Activation of sGC, increased cGMP levels)

Eqn 2. sGC → PKG and/or cGMP-dependent PDEs

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In contrast, endogenous S-nitrosothiols (RSNOS: redox form NO·) and peroxynitrite (OONO·, a potent oxidant formed from the rapid reaction of NO, with superoxide free radical O₂⁻) could potentially exert direct cGMP-independent effects either through S-nitrosylation and/or oxidation reactions at regulatory protein thiol groups (or “redox-switches”).¹¹⁻¹⁴

Eqn 3. RSNO + RS'/rs' → RS'NO (S-nitrosylation of single regulatory thiols)

Eqn 4. RSNO/OONO⁻ + R'S/rs' → RSSR⁻ (Reversible oxidation of regulatory vicinal thiols)

In turn, in addition to ischemic conditions, superoxide O₂⁻ can also be generated from several additional cellular sources, including NAD(P)H oxidases and NOS isoforms themselves.¹⁵⁻¹⁷ Hence, in localized cellular domains where there is relatively high colocalization of expression of NO isoforms and O₂⁻ generating molecules excess OONO⁻ generation may be predicted⁵⁻⁷,¹⁴ and hence direct NO-redox regulatory effects on ion channel function may predominate. In this regard, numerous studies have indicated that NO-related compounds can exert both indirect and direct modulatory effects upon various ion channels expressed in numerous cell types, including NMDA receptors, Ca²⁺-activated K⁺ channels, ryanodine-sensitive sarcoplasmic reticulum Ca²⁺ release channels, various K⁺ channel types and cardiac atrial and ventricular L-type Ca²⁺ channels.¹⁸⁻²⁸ These previous results are significant, since indirect versus direct NO-related pathways could potentially produce opposing regulatory effects on ion channel and overall cardiac myocyte function.²⁻²⁰,²⁸

However, to date it has been difficult to assign specific mechanisms by which distinct cardiac myocyte types and voltage-sensitive ion channels expressed within them could achieve selectivity between indirect versus direct NO- and redox-related signaling mechanisms. This is also true for various ion channels in other cell types that have been studied to date.

Heterogeneous expression patterns of NO-related regulatory enzyme systems. While heterogeneity in expression of voltage-gated ion channels and ancillary subunits among different cardiac myocyte types is now recognized,²⁹⁻³⁵ in many discussions of cardiac function it is nonetheless tacitly assumed that regulatory enzyme systems involved in ion channel modulation are uniformly expressed throughout the different anatomical regions of the heart. However, using immunofluorescent localization we have previously demonstrated in both ferret and human ventricle that Type III NO synthase (eNOS) and sarcoclemmal bound superoxide dismutase (ECSOD) isoforms are heterogeneously expressed, with both enzymes being highly expressed and colocalized in the right ventricle (RV) and subepicardial surface of the left ventricular wall (LV epi) but markedly reduced to absent in the left ventricular subendocardial surface (LV endo).³⁶

These previous findings³⁶ suggested an important functional correlation between the different expression patterns of eNOS and ECSOD in determining the relative magnitude of NO-mediated indirect (sGC-dependent) versus direct (sGC-independent) regulatory effects among LV epi versus LV endo myocytes and ion channels and regulatory subunits expressed within them²⁹⁻³⁵ under both normal and pathological conditions. To be viable, this hypothesis also requires heterogeneous expression levels of NO-activated soluble guanylyl cyclase (sGC) among different LV myocyte types. However, overall relative expression levels of sGC protein among the different major anatomical regions and myocyte types of the heart, and their correlation with heterogeneous expression of various ion channel types, have not been reported.

To begin to address these important issues we have conducted an extensive analysis of the expression patterns of sGC protein in the major anatomical tissue regions and myocyte types of the ferret heart, and a similar but limited analysis in human left ventricular tissue sections. We demonstrate for the first time that sGC is heterogeneously expressed in different cardiac tissue and myocyte types. We further demonstrate, at both the whole tissue and single myocyte levels, that sGC protein gradients virtually overlap both eNOS and ECSOD protein gradients. In turn, these sGC/eNOS/ECSOD gradients close parallel the heterogeneous expression gradients for various Kv channels (including Kv1.4, Kv4.2, Kv4.3 and HERG) and KChIP2 isoforms we have previously demonstrated to exist in the heart.²⁹⁻³¹,³³,³⁴

Our results provide the first demonstration of molecular and cellular mechanisms by which specific cardiac myocyte types could possibly achieve selectivity among indirect versus direct NO- and redox-related signaling pathways involved in regulation of cardiac ion channel function, as well as emphasizing the complexity and heterogeneity of NO-related ion channel regulatory mechanisms in the heart. Our results also provide a relevant framework for understanding the complexity of NO/redox-related effects on ion channels expressed in numerous other cell types.

MATERIALS AND METHODS

Isolated myocytes. All protocols on ferrets (Mustella putorius furo) were conducted in accordance with NIH approved guidelines of the Institutional Animal Care and Use Committee, University at Buffalo, SUNY. Hearts were obtained from 10–16 week-old male ferrets (anaesthetized with 35 mg/kg Na-pentobarbitral intraperitoneal). Single myocytes were enzymatically isolated (Langendorff perfusion, subsequent enzyme treatment [collagenase, protease, elastase]) from selected anatomical regions of the ferret heart (sinoatrial node, right and left atria (entire free wall), right ventricle (entire free wall), left ventricle (entire free wall), and left ventricular subepicardial and subendocardial surfaces (~1 mM tissue strips dissected from each region) exactly as previously described.²⁰,³⁰,³⁶

Ventricular and atrial tissue sections. Ferret heart. Cryosections were obtained from ferret hearts (male, 10-16 weeks-old) initially perfused (Langendorff apparatus) with O₂-saturated control solution (in mM: 144 NaCl, 5.4 KCl, 1 MgSO₄, 1.8 CaCl₂, 10 HEPES, pH = 7.25) and subsequently perfused with the same solution plus 3% paraformaldehyde. Cross sections (6 µM thickness) were then prepared exactly as previously described.²⁰,³⁰,³⁶

Human heart. Human LV epi and LV endo tissue sections were prepared from 3 hearts obtained after rapid autopsy (samples were kindly provided by the late Dr. K.A. Reimer, Department of Pathology and Alzheimers Research Study Group, Duke University). Tissue sections were prepared from the hearts of Caucasian males 23, 48 and 67 years of age, none of who had any known reported clinical history of heart disease. Preparation and analysis were conducted exactly as previously described.³⁶

Soluble guanylyl cyclase antibody and immunoblot analysis. The soluble guanylyl cyclase (sGC) antibody employed was commercially

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obtained (Calbiochem; rabbit polyclonal; reported to recognize the α1 subunit of heterodimeric α/β sGC). Both the eNOS and ECSOD specific antibodies employed and methodologies for their use were as previously described and characterized in detail.36

Preparation of membrane proteins and tissue homogenates. Immuno blot analyses of ferret tissue preparations were conducted exactly as previously described in detail.36,39 Human ventricular tissue samples were obtained upon rapid autopsy and were immediately frozen in liquid nitrogen. Membrane preparations were then obtained as previously described.36 Membrane proteins obtained from various ventricular regions were fractionated on 10% SDS-PAGE gels (Proselve 50% solution, FMC) with appropriate protein markers, followed by transfer to ECL Hybond nitrocellulose membranes (Amersham). The membranes were then first incubated in blocking buffer (1% BSA in PBS-0.1% Tween 20) for 90 minutes, and subsequently incubated with sGC antibody (1:1,000). The membranes were then subsequently incubated with HRP conjugated secondary antibody (anti-rabbit IgG; Jackson Laboratories). After extensive washing the membranes were incubated in ECL solution (Amersham) and signals recorded on ECL hyperfilm.

Immunofluorescence (IF). IF measurements were conducted on myocytes isolated from specific anatomical regions of the ferret heart and tissue sections as previously described30,36 with the following exceptions: the fluorochromes used in the present measurements were Alexa 488 (green), Alexa 555 (red) and Alexa 350 (blue) (Molecular Probes). For colocalization measurements the fluorochromes were directly conjugated with specific antibodies. The sGC primary antibody (1: 700 dilution) was either directly conjugated with Alexa 350 or the secondary antibody (anti-rabbit IgG; Jackson Laboratories) was conjugated with Alexa 488. Slides were scanned using a Zeiss LSM410 confocal microscope, and digitized images were subsequently processed as previously described.30,36

Approximate percent expression levels of sGC among myocyte samples were estimated by visual examination and enumeration as previously described.30,36

Flow cytometry analysis. Fluorescent automated cell sorting (FACS) analysis was performed using a FACScan cytofluorometer and LYSYS II software (Becton Dickinson, San Jose, CA). Preparations of isolated myocytes used for analysis were suspended in PBS plus 1% paraformaldehyde. Measurements were facilitated by adapting a 200 μM nozzle to aid the flow of myocytes without disruption or damage. One x 10⁶ myocytes were analyzed per run. Scanned myocytes were gated to exclude nonmyocyte cell types (e.g., fibroblasts, endothelial cells, neurons) based on forward and side scatter properties of the cells.

Estimation of basal NO production levels. Basal NO production was indirectly measured by reducing nitrite to nitrate by nitrate reductase and Griess reagent (NO Quantitation Kit; Active Motif). Nitrate was colorimetrically quantified at 540 nm. Tissue homogenates obtained from specific regions of the ferret heart were centrifuged and filtered through a 10,000 Dalton micropore filter to remove higher MW proteins. Nitrite and nitrate standard curves were independently obtained on specific tissue homogenates (50 μg). Quantification was subsequently conducted by first determining endogenous nitrite concentration and then total nitrite plus nitrate concentration. Endogenous nitrate concentration was then determined by subtracting nitrite concentration from the total nitrite plus nitrate concentration.

Interpretive limitations of IF results. The specific immunofluorescence patterns which we obtained for any one specific antibody cannot be quantitatively compared to those obtained from another antibody, i.e., only relative comparisons among binding patterns can be made.30,36 Thus, the relative intensity profiles illustrated in Figure 3 are for comparative purposes only in that the intensity values for each enzyme were normalized for that protein by defining the maximum intensity measured in that particular tissue profile as 100%.

RESULTS

Immunoblot analysis. To determine the specificity and appropriateness of the sGC antibody employed immunoblot analysis was initially performed. Protein homogenates were prepared from tissue homogenates from both ferret and human brain (Br) and specific anatomical tissue regions of the ferret and human heart (sinoatrial node [SA], left atrium [LA], right atrium [RA], right ventricle [RV; entire free wall], left ventricle [LV; entire free wall], and specific LV subepicardial [LV epi], LV midventricular [LV mid], and LV subendocardial [LV endo] surfaces).8

Consistent with reported molecular masses of the α1 subunit (~73–82 kD),9,37 in both ferret and human a single prominent antibody binding pattern at ~75 kD was observed in all preparations (Fig. 1). However, among the various ferret protein homogenates, relative binding was most prominent in SA, RA, RV and LV epi but was markedly reduced in LA and LV endo (similar results were obtained from a total of n = 5 protein homogenate samples prepared from n = 5 ferret hearts). Similarly, in the human heart preparations relative binding was most prominent in all preparations except the specific LV endo preparation, wherein binding was markedly reduced (similar results were obtained from n = 3 homogenates prepared from n = 3 human hearts). These results suggested that there might be a marked heterogeneous expression gradient of sGC protein across the LV free wall of both ferret and human hearts.

Figure 1. Immunoblot analysis of sGC antibody specificity and binding patterns. Protein homogenates (50 mg protein/lane) were prepared from indicated anatomical regions of the ferret and human heart. Ferret tissue homogenates. Br, brain; SA, sinoatrial node; LA, left atrium, RA, right atrium, LV, whole left ventricular wall; LV epi, left ventricular subepicardium, LV endo, left ventricular subendocardium, and LV mid, middle region of left ventricular wall. MWM, molecular weight markers. Human homogenates. “H”, human; other designations as per ferret.
**Heterogeneous Expression of Cardiac sGC Protein**

**Ferret ventricular tissue sections. sGC protein expression.** To test the above hypothesis, we conducted IF analysis of basal sGC expression in sagittal ventricular tissue sections (6 μM thickness) prepared from ferret heart. Representative results are illustrated in Figure 2. Positive controls (cardiac specific troponin antibody, direct IF) gave a high and uniform fluorescence in all anatomical regions (Fig. 2B), while negative controls (application of secondary antibody in the absence of primary sGC antibody) failed to produce any significant fluorescence signal (Fig. 2C).

Next we determined overall ventricular sGC antibody binding patterns. A heterogeneous expression pattern was observed, with sGC being highly expressed in the RV and LV epi but markedly reduced to absent in the LV endo and septum (Fig. 2D). Similar IF results were obtained from sagittal ventricular tissue sections prepared from n = 4 ferret hearts. Thus, at the whole ventricular tissue level there is a marked heterogeneous expression gradient of sGC protein across the ferret LV free wall.

**Ventricular tissue sections. sGC colocalization with eNOS and ECSOD.** In our previously proposed dual regulatory model of NO-related indirect versus direct modulation of cardiac ventricular L-type calcium channels we speculated that there may be marked colocalization in the expression patterns of sGC, eNOS and ECSOD protein among different LV myocyte types. Figure 3 illustrates representative IF results obtained on eNOS (red fluorescence), ECSOD (green fluorescence), and sGC (blue fluorescence) expression in adjacent ferret ventricular sagittal tissue sections (each 6 μM thickness). There was a distinct and striking overlap of expression patterns of the three enzymes, as was determined both visually and by comparison of relative intensity profiles through the apical, midventricular and basal regions of the sections. To further characterize this overlap, Figure 4 illustrates results of colocalization measurements. These direct IF measurements verified that there was a marked colocalization of protein expression gradients among the three enzymes, with all being highly expressed in the RV and LV epi but markedly reduced to absent in the LV endo.

Therefore, the expression pattern of sGC virtually overlaps both the eNOS and ECSOD expression gradients.

**Atrial tissue sections. IF measurements of eNOS, ECSOD, and sGC expression in tissue sections prepared from the ferret right atrium (RA; both dorsal and ventral surfaces) and left atrium (LA; dorsal wall; preparation orientation given in Figure 5A–B1 were also conducted. All three atrial preparations gave uniform staining for cardiac specific troponin (Fig. 5D, J and P) and failed to stain after application of labeled secondary antibody in the absence of primary antibody (negative controls; Fig. 5E, K and Q).

There was a marked disparity in the expression levels of the three enzymes between RA and LA. In RA, eNOS, ECSOD, and sGC were all abundantly expressed and colocalized (Fig. 5 F–N). In contrast, in the dorsal wall of the LA expression of all three enzymes was markedly reduced to absent (Fig. 5R, S and T). Hence, similar to the distinct anatomical regions of the ventricle, there is both heterogeneity of expression and marked colocalization of eNOS, ECSOD, and sGC among the right and left atria.

**Isolated myocytes.** The antibody binding patterns observed in whole ventricular and atrial tissue sections reflect not only sGC expression in myocytes but also expression within other nonmyocyte cell types (e.g., endothelium, smooth muscle, neurons). Therefore, to determine if the whole tissue expression gradients were also present at the single myocyte level, IF analysis was next conducted on samples of enzymatically isolated myocytes prepared from the ferret sinoatrial node (SA), RA, RV, LV epi and LV endo. Negative controls (secondary antibody applied in the absence of primary sGC antibody) of single myocyte samples failed to produce any significant fluorescence (Fig. 6A–D). However, in the presence of primary sGC antibody, fluorescence was abundant in the majority of SA, RA, RV, and LV epi myocytes, but was relatively reduced to absent in LV endo myocytes (samples of n > 500 myocytes for each myocyte type obtained from a total of 5 ferret hearts; Fig. 6, lower panels).

As an independent control to our visual analysis of sGC, eNOS, and ECSOD expression in isolated myocytes, we also conducted fluorescence activated cell sorting (FACS) measurements on preparations of myocytes (n = 4) isolated from ferret RV, LV epi, and LV endo (see Methods). The mean percent myocyte expression profiles for eNOS, ECSOD, and sGC based upon this FACS analysis gave results which were essentially identical with the expression patterns determined by visual examination (data not shown).

**Subcellular localization patterns.** Similar to eNOS and ECSOD, confocal optical z-section analysis of specific individual single myocytes indicated that sGC antibody binding was localized to regions adjacent to the sarcolemma (Fig. 7). This may indicate that sGC is selectively bound by intracellular elements so as to localize it near eNOS and/or ECSOD. While at present we do not know the mechanisms underlying this localization, our confocal analysis clearly indicated that all three enzymes colocalize to sarcolemmal and/or immediately adjacent subsarcolemmal domains. Therefore, while we can not attribute the expression gradients of sGC within the whole ventricular and atrial tissue sections exclusively to myocytes, these results demonstrate that expression of sGC in RV, LV epi, and LV endo myocytes closely parallels that observed in the whole tissue sections.

**Human ventricular tissue sections.** The immunoblot results on human heart homogenates (Fig. 1) suggested that there may also be a similar heterogeneous expression gradient of sGC in human heart. We therefore conducted a limited IF analysis of sGC expression in human LV tissue sections (preparation orientation illustrated in Fig. 8A and B). As was observed for ferret LV, sGC expression was relatively high in human LV epi but markedly reduced in LV endo (Fig. 8C–F). Similar results were obtained from a total of n = 3 human hearts. Therefore, the expression gradient of sGC across the LV free wall, at least at the level of whole tissue, is not unique to ferret heart, underscoring the general importance of our findings.

**Functional implications.** Estimated basal NO levels. Basal NO production was indirectly estimated by measuring nitrate levels in tissue homogenates prepared from ferret RA, RV, LV epi, and LV endo. Mean results are illustrated in Figure 9 (RA, n = 5 preparations; RV, LV epi, LV endo, n = 6 preparations). NO levels were highest in LV epi, intermediate in RA and RV and lowest in LV endo. These results positively correlate with the whole tissue expression levels of sGC and eNOS.

**DISCUSSION**

It has proven difficult to determine, under both normal and pathological conditions, the relative contributions of the NO/redox-related indirect (sGC-dependent) versus direct (sGC-independent) signaling pathways to overall regulation of cardiac myocyte and ion channel function (this is also true of other cell and ion channel...
Figure 2. sGC protein expression in ferret ventricular tissue sections. (A) Orientation: Whole ferret heart sagitally bisected. Note that the atria have been removed for clarity. (B) Orientation and positive control: Cardiac specific troponin antibody (direct IF; green fluorescence). AO, aorta; CA, coronary artery; Sep, septum. (C) Negative control: Lack of fluorescence after application of fluorochrome conjugated secondary antibody in the absence of primary sGC antibody. Main panel (D) Overall sGC antibody binding patterns (green fluorescence). Subpanels (D1–D8): 60X enlargements taken from the indicated regions (white boxes).
Heterogeneous Expression of Cardiac sGC Protein

Figure 3. Comparison of eNOS, ECSOD, and sGC protein expression in ferret ventricular tissue sections. Central panels I: (A) eNOS [red], (B) ECSOD [green] and (C) sGC [blue]. Subpanels (A1–C4), 63X magnifications taken from the indicated regions (white boxes). Yellow lines in main panels (A–C) indicate the relative percent intensity of the fluorescence signal measured at the indicated white lines for the apical, midventricular, and basal regions of the ventricle. Scale tick marks correspond to 0%, 50% and 100% relative peak fluorescence for each individual profile.
types). It has also been difficult to assign mechanisms by which cardiac myocytes/ion channels could achieve selectivity between indirect versus direct pathways. Our results are thus significant since they provide the first demonstration of distinct molecular and cellular mechanisms for potentially generating such NO/redox-related selectivity among different tissues, myocyte types and ion channels in the heart. In particular, under normal physiological conditions superoxide free radical \( \text{O}_2^\cdot \) can be generated from numerous metabolic sources, including the activities of membrane-bound NAD(P)H oxidases and NOS isoforms themselves.\(^{5,15-17}\) In turn, free radical \( \text{NO}^\cdot \) can very rapidly react (4–7 \( \times \) \( 10^9 \)/M-sec) with \( \text{O}_2^\cdot \) to form \( \text{OONO}^\cdot \) (peroxynitrite; Eqns. 3 and 4), a potent thiol oxidant, which when subsequently protonated (\( \text{pK}_a = 6.5 \)) can dissociate to form the very toxic hydroxyl free radical \( \text{OH}^\cdot \) and nitrogen dioxide \( \text{NO}_2^\cdot \).\(^{5-7,14}\)

It has been previously hypothesized that when \( \text{NO}^\cdot \) and \( \text{O}_2^\cdot \) are simultaneously generated in closely adjacent cellular domains free radical \( \text{NO}^\cdot \) would be rapidly "scavenged" and subsequent formation of \( \text{OONO}^\cdot \) would become dominant.\(^{14,36}\) However, the simultaneous expression of ECSOD within the same adjacent sarcolemmal and extracellular domains would help counter \( \text{OONO}^\cdot \) formation, thus increasing free localized \( \text{NO}^\cdot \) concentration. This latter proposal\(^{14,36}\) is strengthened by the fact that ECSOD can catalyze the dismutation of \( \text{O}_2^\cdot \) at a rate (~1 \( \times \) \( 10^9 \)/M-second) which can very effectively compete with \( \text{NO}^\cdot – \text{O}_2^\cdot \) interactions.\(^{5-7,14}\) Thus, in cellular regions where both membrane bound eNOS and ECSOD are colocalized\(^{14,36}\) net free \( \text{NO}^\cdot \) formation would become dominant, allowing generation and/or predominance of indirect (cGMP-dependent) regulatory effects. Our present findings that NO-activated heterogeneous sGC expression levels in the heart very closely overlap those of both eNOS and ECSOD provides new and very strong supportive evidence in favor of this model. Within such a framework, our results imply that NO-related indirect effects (Eqns. 1 and 2) would be prominent in RA, RV and LV epi myocytes, while direct (cGMP-independent) effects (S-nitrosoylation, vicinal thiol oxidation, and/or OONO-mediated oxidation effects; Eqns. 3 and 4)\(^{6,7,11-13,38}\) would be predominant in LA and LV endo myocytes (for experimental demonstrations of opposing NO-related indirect versus direct regulatory effects on the L-type \( \text{Ca}^{2+} \) current in ferret RV myocytes see ref. 20).

**Implications for previous cardiac myocyte ion channel studies.** Our results indicate that the frequently held assumption that regulatory enzyme systems involved in ion channel modulation are uniformly expressed throughout all of the different myocyte types of the heart is incorrect. They also stress the importance of paying very careful attention to both tissue and specific myocyte types when analyzing cardiac myocyte ion channel function and associated regulatory mechanisms. Hence, the expression gradients that we have discovered should provide a rationale basis for understanding at least some of the considerable variability in reported effects of various N-oxides on cardiac myocyte and ion channel function. To cite one specific example, the fact that both eNOS and sGC protein expression are heterogeneous may provide insights into the question over the obligatory versus non-obligatory involvement of NO-production in cholinergic regulation of the L-type \( \text{Ca}^{2+} \) current \( I_{\text{Ca,L}} \) in different cardiac myocyte types.\(^{39-47}\) In this regard, it is interesting to note that while the majority of ferret right ventricular myocytes express eNOS, ECSOD, and sGC, in a previous study we were unable to obtain any evidence for obligatory NO-production in muscarinic-mediated inhibition of the basal L-type calcium current in these specific myocyte types.\(^{48}\) Our present results also have implications for \( \alpha \)-adrenergic signaling mechanisms in different anatomical regions of the heart, mechanisms that have been suggested to involve \( \text{NO}^\cdot \) and sGC-mediated signaling mechanisms (e.g., feline atrial myocytes).\(^{22}\)

It is also very interesting to note that the expression gradients of sGC, eNOS, and ECSOD across the LV free wall also very closely parallel the expression gradients of specific Kv channel \( \alpha \) subunits underlying generation of distinct repolarizing \( K^+ \) currents, including the rapidly inactivating ERG-mediated \( I_{\text{Kr}} \) and the two major distinct transient outward \( K^+ \) current \( (I_{\text{to}}) \) phenotypes, \( I_{\text{to,slow}} \) and \( I_{\text{to,fast}} \).\(^{29,30} \) The latter two \( I_{\text{to}} \) phenotypes are functionally important in early rapid (“phase 1”) repolarization and frequency-dependent modulation of action potential morphology in distinct ventricular myocyte types.\(^{29}\) Shal family Kv4.2 and/or Kv4.3 \( \alpha \) subunits, which are responsible for generation of the rapidly recovering \( \text{Heteropoda} \) toxin (HPTX)-sensitive \( I_{\text{to,fast}} \) phenotype,\(^{29,30,50,51} \) are highly expressed in ferret RV and LV epi myocytes,\(^{29,30} \) i.e., in myocyte types where sGC, eNOS and ECSOD expression are all high.\(^{36}\) In contrast, Shaker family Kv1.4 \( \alpha \) subunits, which are responsible for the slowly recovering HPTX-insensitive \( I_{\text{to,slow}} \) phenotype,\(^{29} \) are highly expressed in LV endo myocytes,\(^{29,30} \) i.e., in myocyte types where sGC, eNOS and ECSOD expression are all relatively low.\(^{36}\) It is thus intriguing to note that the inactivation characteristics of both Kv1.4 and Kv4 channels have been demonstrated to be selectively modulated by alterations in redox-status and redox-related metabolites.\(^{29} \)

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Image 4. Ventricular eNOS, ECSOD and sGC colocalization patterns. (A) eNOS (red) and ECSOD (green). Colocalization = yellow. (B) ECSOD (green) and sGC (blue). Colocalization = cyan. (C) eNOS (red) and sGC (blue). Colocalization = purple. (D) Net colocalization of all three enzymes. Colocalization = white to light violet.
Figure 5. sGC expression in ferret atrial tissue sections. (A) Orientation: Basal region of the ferret heart exposing the AO and superior (SVC) and inferior (IVC) vena cava. Note that the ventricle has been removed for clarity. Black arrow indicates region where cut was made to expose the RA. The yellow line in (A1) and (A2) indicate the region from which sections were obtained. CT, crista terminalis. (B) LAA, left atrial appendage. Yellow line in panel (B1) indicates the region from which LA tissue sections were obtained. (C–H) Dorsal wall of RA; (I–N) ventral wall of RA; and (O–T) dorsal wall of LA. (C, I and O) are transmitted light micrographs of tissue preparations. (D, J and P) Cardiac specific troponin antibody (direct IF, green fluorescence). (E, K and Q) Lack of fluorescence after application of secondary antibody in the absence of primary antibody. Main panels (F, L and R) eNOS (red); (G, M and S) ECSOD (green); and (H, N and T) sGC (blue). Adjacent subpanels are 60X enlargements taken from the indicated regions (white boxes) of the corresponding main panels.
Figure 6. sGC expression in ferret isolated cardiac myocytes and comparison to myocyte eNOS and ECSOD expression. Upper panels (A–H) Expression patterns (green fluorescence) in samples of myocytes viewed in a single field of exposure (20X magnification). Myocytes were isolated from right atrium (RA), right ventricle (RV), left ventricular subendocardium (LV en) and left ventricular subepicardium (LV ep). (A–D) Negative controls (con), i.e., myocytes incubated only with fluorochrome conjugated secondary antibody. (E–H) sGC antibody binding patterns (green fluorescence). Lower panels: Percent expression of eNOS, ECSOD, and sGC in myocytes isolated from ferret sinoatrial node (SA), RA, LV endo and LV epi. Main color panels illustrate representative positive fluorescence results (eNOS, red; ECSOD, green; sGC, blue) obtained from indicated myocyte type. Adjacent bar graphs give mean percent expression (± SEM) of eNOS, ECSOD and sGC in specific myocyte types determined by visual enumeration.
It is also now established that a family of calcium-sensing potassium channel interacting proteins (KChiPS) are importantly involved in selective modulation of the inactivation and recovery characteristics of Kv4.2/4.3 channels that underlie generation of native LV I_{to,fast}. It is again intriguing to note that the expression gradients of KChiP2 isoforms across the ferret LV free wall also closely parallel the expression gradients of sGC, eNOS and ECSOD.

While at present these results on various regulatory protein expression gradients in the ventricle are only correlative, and demonstration of their functional consequence will require future patch clamp and molecular biological analyses, based upon their striking overlap it is nonetheless reasonable to suggest that heterogeneous expression gradients of sGC, eNOS and ECSOD may provide an important but presently unrecognized coupled system for selective NO- and/or redox-related modulation of excitation-contraction coupling, repolarization and frequency-dependent action potential characteristics of specific ventricular myocyte types.

**Type I nNOs.** Type I nNOS expression has been previously detected in cardiac myocytes, wherein it appears to be localized to the sarcoplasmic reticulum. Our previous results also indicate that type I nNOS is also expressed in ferret heart. While our present results do not allow us to conclude that it is specifically localized to the SR membrane, our observations do indicate that it is expressed intracellularly. However, what is even more interesting, from a potential functional point of view, is our observation that Type I nNOS expression is almost exclusively localized to LV endo, i.e., its expression gradient is virtually a “mirror image” of that of sGC, eNOS and ECSOD (refer to Fig. 3 in ref. 36). The presence of this NOS isoform probably contributed to our estimated measurements.

Figure 7. Confocal analysis of subcellular localization patterns of eNOS, ECSOD, and sGC in ferret LV epi myocytes. Subpanels indicated 1–8 illustrate optical Z sections taken at successive 0.5 μM intervals through the width of the myocytes. [A1–A8] eNOS (red), [B1–B8] ECSOD (green), [C1–C8] sGC (blue) and [D1–D8] colocalization of all three enzymes. Note the sarcolemmal localization patterns when the Z section was through the approximate middle of the myocytes (subpanels 4 and 5 for each condition). (A–D) (right) illustrate the overall summed fluorescent signals for each series of optical Z sections.
Figure 8. Comparison of eNOS, ECSOD, and sGC expression in human and ferret left ventricular tissue cross sections. sGC (blue), eNOS (red), ECSOD (green), colocalization of all three enzymes (white). (A–J) human heart: (A and B) Section orientation. White line in (A) corresponds to the transverse section, while (B) illustrates the transversely bisected heart. White boxes in (B) indicate regions from where sections were obtained. AO, aorta, LA, left atrium, RA, right atrium, LV ep, left ventricular subepicardium, LV en, left ventricular subendocardium. (C, E, G and H) LV subepicardium. (D, F, H and J) LV subendocardium. (K–T) ferret heart: (K and L) section orientation. Green fluorescence in (L) corresponds to binding of cardiac troponin C antibody. (M, O, Q and S) LV subepicardium. (N, P, R and T) LV subendocardium.

Figure 9. Potential functional implications. Estimated basal NO concentrations in ferret RA, RV, LV endo, and LV epi cardiac tissue homogenates. Mean values from: RA, n = 5 preparations; RV, LV epi, LV endo, n = 6 preparations. See text for further details.
basal NO levels in LV endo tissue. The functional implications of this heterogeneous nNOS expression are presently unclear. In light of our present results on the relative lack of sGC expression in LV endo myocytes, the expression of nNOS in this tissue may indicate involvement of this NOS isoform in modulation of specific direct cGMP-independent functions, e.g., regulation of sarcoplasmic reticulum Ca2+ release channels.

Summary. Our results are the first demonstration in the mammalian heart of: (i) heterogeneous expression gradients of NO-activated sGC protein; (ii) marked overlap of sGC gradients with both eNOS and ECOSD protein gradients; and (iii) positive correlations of such expression gradients with both estimated basal NO production levels and heterogeneous expression gradients of various Kv channel types (ERG, Kv1.4 and Kv4.2/4.3) and KCChIP2 isoforms among different cardiac tissue and myocyte types.

In combination with previous results indicating marked localization of NO2–36,55 and superoxide oxide dismutase isoforms36 in cardiac myocytes, our results provide very strong suggestive evidence for coupled (“crossed-talking”) enzymatic mechanisms by which different cardiac myocyte and ion channel types expressed within them could achieve selectivity between the various NO/redox-related signaling pathways. We propose that colocalized expression of sGC, eNOS and ECOSD would allow indirect (cGMP-dependent) NO-related modulatory effects to be prominent in SA, RA, RV and LV epi myocytes, while low expression of these same enzymes would allow direct (cGMP-independent) effects to be prominent in LA and LV endo myocytes.

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