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The role of nitric oxide and reactive oxygen species in the positive inotropic response to mechanical stretch in the mammalian myocardium

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ABSTRACT

The endothelial nitric oxide synthase (eNOS) has been implicated in the rapid (Frank–Starling) and slow (Anrep) cardiac response to stretch. Our work and that of others have demonstrated that a neuronal nitric oxide synthase (nNOS) localized to the myocardium plays an important role in the regulation of cardiac function and calcium handling. However, the effect of nNOS on the myocardial response to stretch has yet to be investigated. Recent evidence suggests that the stretch-induced release of angiotensin II (Ang II) and endothelin 1 (ET-1) stimulates myocardial superoxide production from NADPH oxidases which, in turn, contributes to the Anrep effect. nNOS has also been shown to regulate the production of myocardial superoxide, suggesting that this isoform may influence the cardiac response to stretch or ET-1 by altering the NO-redox balance in the myocardium. Here we show that the increase in left ventricular (LV) myocyte shortening in response to the application of ET-1 (10 nM, 5 min) did not differ between $nNOS^{-/-}$ mice and their wild type littermates ($nNOS^{+/+}$). Pre-incubating LV myocytes with the NADPH oxidase inhibitor, apocynin (100 μM, 30 min), reduced cell shortening in $nNOS^{-/-}$ myocytes only but prevented the positive inotropic effects of ET-1 in both groups. Superoxide production (O_2^-) was enhanced in $nNOS^{-/-}$ myocytes compared to $nNOS^{+/+}$; however, this difference was abolished by pre-incubation with apocynin. There was no detectable increase in O_2^- production in ET-1 pre-treated LV myocytes. Inhibition of protein kinase C (chelerythrine, 1 μM) did not affect cell shortening in either group, however, protein kinase A inhibitor, PKI (2 μM), significantly reduced the positive inotropic effects of ET-1 in both $nNOS^{+/+}$ and $nNOS^{-/-}$ myocytes. Taken together, our findings show that the positive inotropic effect of ET-1 in murine LV myocytes is independent of nNOS but requires NADPH oxidases and protein kinase A (PKA)-dependent signaling. These results may further our understanding of the signaling pathways involved in the myocardial inotropic response to stretch.

1. Introduction

Mechanical stretch is an important physiological and pathological stimulus in the heart. It is well established that mechanical stretch enhances myocardial contractility [1] but the underlying mechanisms are not completely understood. Under physiological conditions, the myocardial response to stretch is biphasic and involves a large and rapid (within a beat) increase in inotropy, *i.e.*, the Frank–Starling response, which is followed by a slower and a less pronounced increase in the force of contraction, *i.e.*, the Anrep effect. The rapid increase in contraction occurs as a result of a length-dependent increase in cross-bridge formation and myofilament Ca^{2+} sensitivity whereas the slow rise in force that follows is associated with an increase in the amplitude of the intracellular Ca^{2+} transients [2]. Sustained mechanical stretch stimulates the release of angiotensin II (Ang II) from the ventricular myocardium which, in turn, contributes to the Anrep effect *via* a mechanism that involves the release of endothelin 1 (ET-1) [3–5]. It has been postulated that an increase in Ca^{2+} entry *via* the Na^+–Ca^{2+} exchanger (NCX) following activation of the Na^+–H^+ exchanger (NHE) may play an important role in mediating the Anrep effect [3–6]. Indeed, several investigators [4–6] have shown that in trabeculae, papillary muscles or isolated ventricular myocytes from rat, cat or humans, inhibition of NHE blunted the stretch-mediated increase in intracellular Na^+_i as well as its positive inotropic effect. Similarly, inhibition of the reverse mode of the NCX abolished the increase in intracellular Ca^{2+}_i without affecting the increase in intracellular Na^+_i [4] (Fig. 1).

Mechanical stimuli (e.g., circumferential or longitudinal stretch) have been shown to stimulate nitric oxide (NO) release from both the vascular endothelium and LV myocytes [7–8] with potentially important consequences in the regulation of LV function [8,9]. In particular, stimulation of NO production from the coronary endothelium or intracoronary infusion of NO donors has been shown to hasten LV relaxation and reduce LV end-diastolic stiffness, which in turn may influence the Frank–Starling response by increasing LV end-diastolic volume and the length of the myocardial muscle fibers [10]. Conversely, inhibition of NO synthesis attenuates the rise in cardiac

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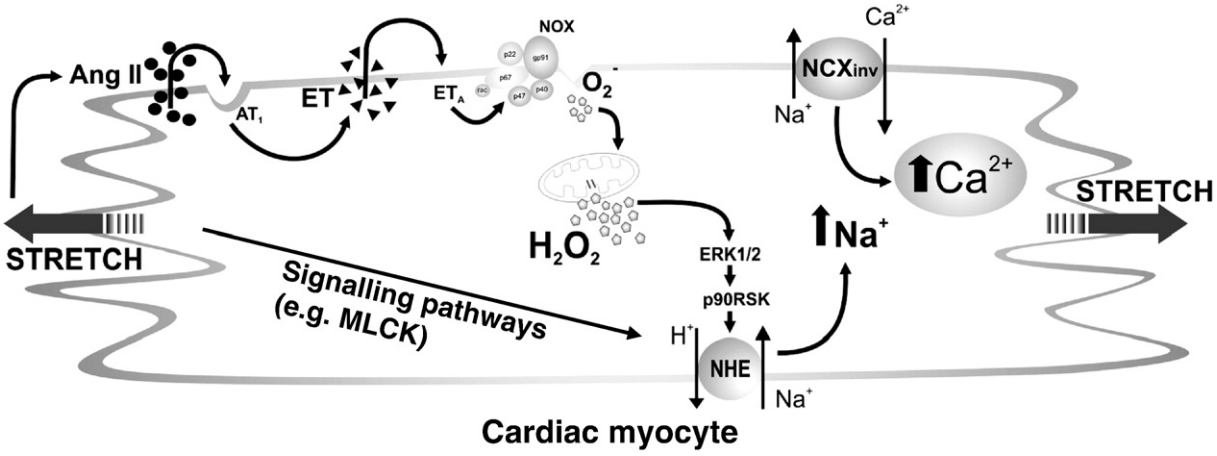

Fig. 1. Schematic illustration of the mechanisms involved in the slow inotropic response to stretch in cardiac myocytes. Sustained stretch induces the release of preformed angiotensin II (Ang II). Cardiac AT1 receptor stimulation promotes the release of endothelin-1 (ET-1). Downstream of ET-1_A receptors, activation of NADPH oxidases produces reactive oxygen species (ROS). Redox-dependent stimulation of Na^+–H^+ exchanger (NHE) leads to an increase in intracellular Na^+ concentration which in turn increases intracellular Ca^{2+} *via* the reverse mode of the Na^+–Ca^{2+} exchanger. Mechanical stretch may also directly activate the signaling pathways (e.g. myosin light chain kinase, MLCK) that mediate slow force response to stretch of myocardium. Modified from Caldiz et al. with permission.

output in response to increases in LV preload in isolated guinea-pig hearts [9] suggesting that the stretch-mediated stimulation of constitutive NO production in the myocardium contributes to the ability of the heart to adjust cardiac output to meet the demand posed by increasing LV filling pressure.

Myocardial production of NO by the endothelial nitric oxide synthase (eNOS) has been implicated in the Anrep effect. Petroff et al. [8] have shown that the increase in Ca^{2+} transient amplitude observed in rat LV myocytes subjected to sustained longitudinal stretch whilst embedded in agarose is associated with an increase in NO production and is abolished by NOS inhibition or by selective disruption of the eNOS gene [8]. The stretched myocytes exhibited an increase in Ca^{2+} spark frequency in the absence of changes in sarcoplasmic reticulum (SR) Ca^{2+} content. These data suggest that stretch-induced NO release may increase contraction by increasing RyR open probability and SR Ca^{2+} release. However, other investigators have shown that the slow increase in contraction in response to stretch in rat trabeculae is maintained even after SR Ca^{2+} release has been disrupted [11] and that non-isoform specific inhibition of NOS had no impact on the positive inotropic effect of longitudinal stretch in rat LV myocytes [12].

A neuronal isoform of NOS (nNOS) has been identified in the mammalian myocardium [13]. In contrast with the myocardial and vascular eNOS isoform, which is mostly localized to plasmalemmal microdomains named caveolae, nNOS has been co-localized with RyR Ca^{2+} release channels in the cardiac SR [14]. Our work [15–17] and that of others [18–20] show that acute nNOS-specific inhibition or targeted disruption of the nNOS gene ($nNOS^{-/-}$) results in an enhanced left ventricular (LV) contractility *in vitro* and *in vivo*, which appears to be driven by a higher Ca^{2+} entry through the plasmalemma Ca^{2+} current (I_{Ca}) [16]. nNOS disruption also results in severely impaired myocardial relaxation both *in vivo* [17,19] and in isolated myocytes [15,16,20]. We have shown that a reduction in the PKA-dependent phosphorylation of phospholamban, secondary to an increase in protein phosphatase activity, accounts for the slower SR Ca^{2+}-reuptake and impaired relaxation in the myocardium of $nNOS^{-/-}$ mice [21]. nNOS-derived NO may also regulate RyR function by modulating the channel's open probability and thus the diastolic "leak" of Ca^{2+} from the SR [22]. These findings suggest that changes in the expression and activity of myocardial nNOS may regulate the myocardial response to stretch, directly through the effect of NO on Ca^{2+} handling proteins [16,21].

Emerging evidence has associated mechanical stretch with the local formation of reactive oxygen species (ROS) in the myocardium [23–25]. At low concentrations, ROS appear to modulate cell signaling chiefly by modifying key thiol groups on proteins that possess regulatory functions [26–28]. For instance, hydrogen peroxide transiently inhibits protein tyrosine phosphatase activity through the reversible oxidation of catalytic cysteines [27]. A number of studies over the last decade have indicated that NADPH oxidases are the main source of ROS involved in myocardial redox signaling and stretch responses [24,25]. NADPH oxidase in cardiac myocytes comprises of a catalytic unit $gp91^{phox}$ (termed Nox2) that forms a heterodimer with $p22^{phox}$ subunit in the membrane and cytosolic regulatory subunits $p47^{phox}$ and $p67^{phox}$ and the small GTP-binding protein Rac1 [29]. Stretch triggers the release of Ang II and ET-1 from the myocardium, which in turn stimulates the activity of NOX2-NADPH oxidases [23,25] *via* a protein kinase C (PKC)-mediated phosphorylation of the oxidase's $p47^{phox}$ subunit, leading to its translocation to the membrane-bound cytochrome (*i.e.*, to the NOX2 and $p22^{phox}$ complex) [29]. This mechanism accounts for the increase in superoxide production in response to Ang II in a number of preparations [30–32]. Recent evidence has also shown that Rac1-GTPase is rapidly activated by both stretch [33,34] and Ang II in ventricular myocytes [35], implicating an early role of Rac1 in mechanotransduction and neurohumoral signaling in the myocardium. Taken together these data suggest that sustained stretch may lead to Ang II-mediated $p47^{phox}$ phosphorylation, transcriptional upregulation of the NADPH oxidase subunits, and activation of Rac1-GTPase, all of which would be expected to increase local ROS-mediated signaling (Fig. 1).

Recent evidence shows that inhibition of nNOS increases myocardial superoxide production [36], suggesting that nNOS may play a novel role in the regulation of myocardial response to stretch indirectly through its alteration of myocardial ROS formation. In this study, we demonstrate that increased activity of NADPH oxidase contributes to enhanced LV myocyte contraction in $nNOS^{-/-}$ mice and that PKA acts as downstream of ROS in the mechanism of mechanical stretch regulation of myocardial contraction.

2. Materials and methods

2.1. Animals

Mice (2–5 months old) homozygous for targeted disruption of nNOS ($nNOS^{-/-}$) were compared to their age-matched wild type littermates ($nNOS^{+/+}$). All protocols were in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act, 1986.

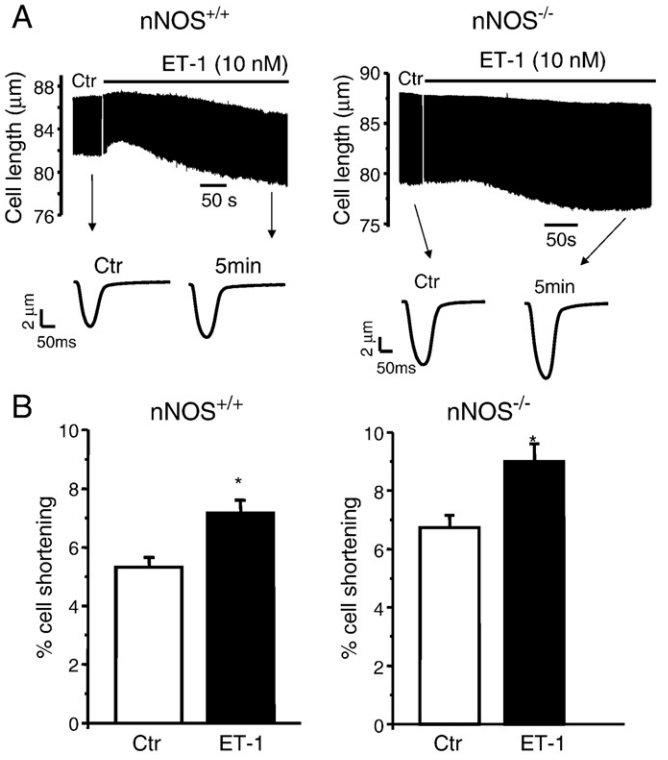

Fig. 2. Effects of ET-1 on cell shortening in LV myocytes isolated from nNOS$^{-/-}$ mice and their wild type littermates (nNOS$^{+/+}$). (A) Continuous recordings of field stimulated (3 Hz) myocyte shortening under control conditions (Ctr) and during the application of 10 nM endothelin 1 (ET-1) and representative individual recording under control conditions and at about 5 min (steady state) after perfusion with ET-1. ET-1 induced an initial transient negative inotropic effect followed by an increase in cell shortening in both genotypes. (B) Mean values of % cell shortening under controls conditions and in the presence of ET-1 (steady state). *P<0.05 *vs.* control.

2.2. Isolation of left ventricular myocytes from mice heart

Left ventricular (LV) myocytes were isolated using a standard enzymatic dispersion technique as described elsewhere [16]. Briefly, the heart was initially perfused for 3 min with a nominally Ca^{2+}-free solution (in mM; NaCl 130, KCl 5.4, $MgCl_2$ 3.5, glucose 10, Hepes 5, Na_2HPO_4 0.4, taurine 20 at a pH of 7.4), followed by a further 9 min with the same solution with enzymes added (collagenase, 1 mg/ml, Worthington Biochemical Co., protease, 0.133 mg/ml, Sigma Aldrich; 1.65 mg/ml BSA; Ca^{2+} 0.05 mM). The LV free wall was isolated and placed in a separate flask containing fresh collagenase-only solution. Myocytes were harvested following further five and ten minute digestion periods, washed twice and re-suspended in storage solution (in mM; NaCl 120, KCl 5.4, $MgSO_4$ 5, $CaCl_2$ 0.2, Na-pyruvate 5, glucose 20, taurine 20, Hepes 10, pH 7.4, NaOH). The myocyte suspension was stored at room temperature and cells were used within 8 h of isolation.

2.3. Measurement of LV myocyte contraction

Isolated LV myocytes were superfused with a solution containing (in mM) NaCl 140, KCl 5.4, $MgCl_2$ 1.2, HEPES 5, Glucose 10, $CaCl_2$ 1.4 at a pH of 7.4. Cell length was measured in LV myocytes field stimulated at 3 Hz by using a video-edge detection system (IonOptix Corp). Cell shortening relative to diastolic cell length (% cell shortening) was compared in LV myocytes from nNOS$^{-/-}$ mice and their wild type littermates. Measurements from at least 10 steady state contractions were averaged for each cell for each stage of the experimental protocols. All experiments were carried out at 35 ± 1.5 °C.

2.4. Measurement of superoxide production in LV myocytes

Superoxide production was measured by using lucigenin (5 μM)-enhanced chemiluminescence in LV myocyte lysates from nNOS$^{-/-}$ mice and their wild type littermates using a single vial luminometer (Berthold FB12) modified to maintain the sample temperature at 37 °C, (as described and validated previously, [37]). Briefly, aliquots of isolated myocytes, which had been incubated at 37 °C in the storage solution with or without ET-1 (10 nM), were homogenized by a freezing–thawing method and transferred into scintillation vials containing 800 μl Krebs-Hepes buffer (in mmol/l: NaCl 118, HEPES 10, $NaHCO_3$ 25, Glucose 5.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.1, $CaCl_2$ 1.5 at pH 7.4 after 60 min aeration with 95%O_2 and 5%CO_2) and 5 μM lucigenin. After 1 min of acclimatisation, the chemiluminescence was measured for a further 10 min. The level of chemiluminescence during the final 1.5 min was determined after subtraction of the background chemiluminescence of the lucigenin-containing buffer.

2.5. Chemicals

Endothelin-1 (ET-1, 10 nM, Merck Biosciences) was used to stimulate NADPH oxidase activity. Membrane permeable amide (14-22, PKI, 2 μM, Merck Biosciences) was used to inhibit the activity of PKA. Chelerythrine (1 μM, Sigma) was used to inhibit the activity of PKC. Apocynin (100 μM, Sigma) was used to inhibit NADPH oxidase activity.

2.6. Statistics

Data are expressed as mean ± s.e.m. and n indicates the number of LV myocytes used. For all comparisons, cells were obtained from a minimum of two hearts per genotype per protocol. Data were analyzed using *ANOVA* (Microsoft Excel). A value of P<0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Role of NO and ROS downstream signaling in regulating myocardial contraction and stretch responses

3.1.1. Role of nNOS-derived NO

Although eNOS-derived NO has been implicated in both the rapid and slow inotropic response to stretch [8,9], this concept has been challenged by more recent reports that failed to observe an effect of non-isoform specific NOS inhibition on stretch-induced inotropy in cardiac tissue or myocytes [6,12]. Whether nNOS-derived NO plays a role in mechanotransduction and myocardial signaling downstream of stretch and ET-1 receptor stimulation has not yet been investigated. We compared the inotropic response to ET-1 (10 nM) in LV myocytes isolated from nNOS$^{-/-}$ mice and their wild type littermates (nNOS$^{+/+}$).

As shown in Fig. 2A, ET-1 affected field stimulated cell shortening (3 Hz, 35 °C, as described in [21]) in nNOS$^{+/+}$ LV myocytes in a time-dependent manner. A small negative inotropic effect (at about 1 min after the perfusion of ET-1) preceded an increase in cell shortening in most of the cells studied. The positive inotropic effect of ET-1 reached a plateau at 5–10 min. These results are in agreement with previous evidence indicating that ET-1 exerts a biphasic inotropic response in guinea-pig or cat cardiac myocytes [38,39]. On average, % cell shortening in LV myocytes from nNOS$^{+/+}$ mice increased from 5.3 ± 0.3% to 7.1 ± 0.4% 5 min after perfusion with a solution containing ET-1 (P<0.001, n = 24).

The inotropic effect of ET-1 did not differ in LV myocytes from mice with selective deletion of the nNOS gene (Fig. 2B); after 5 min the absolute increase in cell shortening induced by ET-1 was 1.8 ± 0.4 μm in nNOS$^{+/+}$ (n = 24) and 2.2 ± 0.4 μm in nNOS$^{-/-}$ (n = 15, P = 0.4

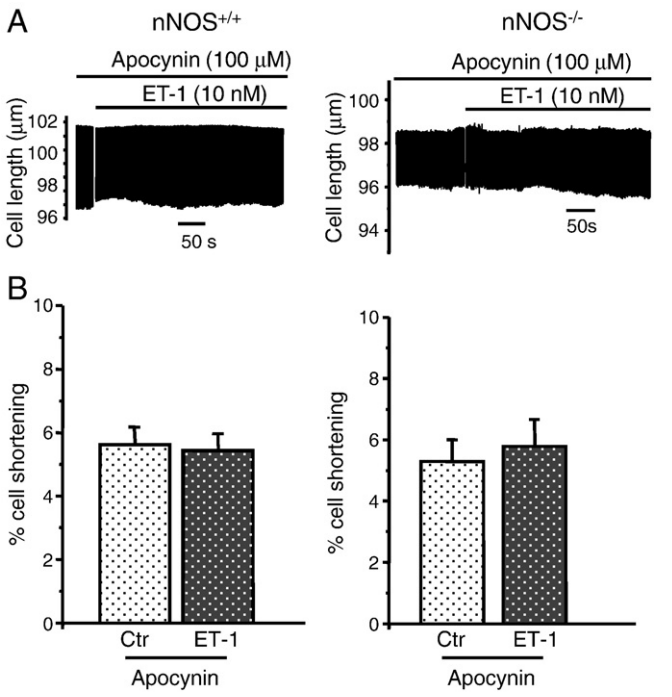

Fig. 3. Effect of NADPH oxidase inhibition with apocynin on basal and ET-1 inotropy. (A) Continuous recordings of field stimulated (3 Hz) myocyte shortening in the presence of apocynin (100 μM) and ET-1. ET-1 (10 nM) failed to induce a positive inotropic effect in both $nNOS^{+/+}$ and $nNOS^{-/-}$ LV myocytes pre-incubated with apocynin. NADPH oxidase inhibition caused a significant reduction in cell shortening in $nNOS^{-/-}$ mice but not in their wild type littermates. (B) In the presence of apocynin, mean cell shortening did not differ between genotypes and between control and ET-1.

between the two groups). As shown previously, basal contraction in $nNOS^{-/-}$ myocytes was greater than in their wild type littermates ($P=0.01$). These data suggest that nNOS-derived NO is unlikely to play an important role in the inotropic response to ET-1 in murine LV myocytes.

It is important to note that the role of nNOS in stretch-induced slow force response is still an open question. The release of Ang II and ET-1 may have been shown to be entirely responsible for stretch-increased force in some studies, e.g. [4]; but others failed to detect the involvement of these neurohormones in the slow response to stretch [6,40]. In these studies, the slow force response to stretch of the human ventricular myocardium was unaffected by the inhibition of Ang II and ET-1 receptors; whereas it responded to the activation of myosin light chain kinase (MLCK, [40]), as discussed below). Whether or not nNOS is involved in MLCK signaling under stretch and regulates force generation warrants further investigation.

In contrast to our findings, Nishimaru et al. have recently shown that ET-1 induces a negative inotropic effect in murine LV myocytes [41]. The reason for this discrepancy is not clear and may be due to differences in experimental conditions (e.g. notably in field stimulation frequency, 0.5 Hz in Nishimaru et al. *vs.* 3 Hz in our study). Stimulation of NHE activity by ET-1 may shift the inotropic response from negative to positive, as shown in studies where NHE inhibition was associated with a negative inotropic response to ET-1 [38,42]. Differences in NHE expression or activation between mice strains may therefore contribute to the variability in the ET-1 inotropic response.

3.1.2. Role of NADPH oxidase activity and ROS

To investigate the involvement of NADPH oxidase in the inotropic response to ET-1, LV myocytes were incubated for 30 min in perfusion solution in the presence or absence 100 μM apocynin, a potent NADPH oxidase inhibitor. Fig. 3 shows that the positive inotropic effect of ET-1 (10 nM) is totally prevented by pre-incubation with apocynin both in $nNOS^{-/-}$ and $nNOS^{+/+}$ myocytes, suggesting that myocardial NADPH oxidases are instrumental in mediating the inotropic response to ET-1 in the murine LV myocardium, irrespective of nNOS.

Apocynin significantly reduced basal cell shortening in LV myocytes from $nNOS^{-/-}$ mice but not in their wild type littermates (from $6.7\pm0.4\%$ under control conditions to $4.8\pm1.6\%$ in the presence of apocynin in $nNOS^{-/-}$, $P<0.01$, $n=15$ and 10 and from $5.3\pm0.3\%$ to $5.6\pm0.5\%$ in $nNOS^{+/+}$, $P=0.6$, $n=24$ and 12, respectively).

These data suggest that stimulation of myocardial superoxide production [20,36,43] by NADPH oxidases may play an important role in mediating the increase in basal inotropy observed in the presence of nNOS gene deletion or inhibition.

Next, we compared superoxide release using lucigenin-enhanced chemiluminescence in LV myocytes from $nNOS^{+/+}$ and $nNOS^{-/-}$ mice that had been pre-incubated in the presence or absence ET-1 and then lysed. Apocynin (100 μM, 10 min) was then applied to the LV myocyte lysates to assess the contribution of NADPH oxidases.

Fig. 4A shows that basal superoxide production was not affected by apocynin application in LV myocyte lysates from $nNOS^{+/+}$ mice, suggesting that under basal conditions, NADPH oxidase activity in wild type LV myocytes must be very low. Surprisingly, ET-1 did not cause a significant increase in superoxide production in these cells (Fig. 4A).

Superoxide production has previously been shown to be increased in intact LV myocytes [43], LV homogenates [20] or tissue chunks from $nNOS^{-/-}$ mice [36]. In agreement with these data, we found a significant increase in basal superoxide production in LV

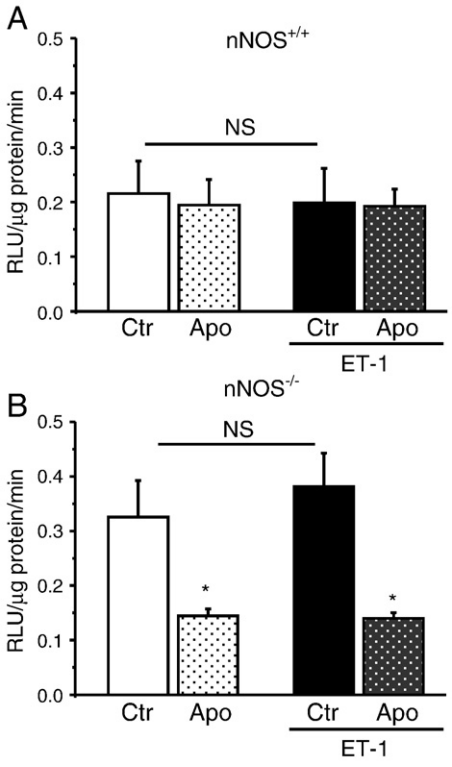

Fig. 4. Measurement of superoxide production using lucigenin (5 μM)-enhanced chemiluminescence in LV myocyte homogenates from $nNOS^{+/+}$ and $nNOS^{-/-}$ mice. ET-1 (10 nM) was pre-incubated for 5 min at 37 °C and LV myocytes were homogenized using a freeze–thaw method (repeated 6 times). (A) Superoxide release did not differ between control and ET-1 treated $nNOS^{+/+}$ myocytes. Application of apocynin (100 μM) did not affect superoxide release suggesting that NADPH oxidase activity is undetectable under basal conditions. (B) Basal superoxide release was greater in $nNOS^{-/-}$ than in $nNOS^{+/+}$ myocytes. Apocynin significantly reduced superoxide production suggesting that NADPH oxidase activity is enhanced in the absence of nNOS. ET-1 also failed to increase superoxide production in $nNOS^{-/-}$ myocytes. *$P<0.05$ *vs.* control.

myocyte lysates from nNOS$^{-/-}$ mice (Fig. 4B). Inhibition of NADPH oxidase activity caused a significant reduction in superoxide production in the nNOS$^{-/-}$ myocardium (Fig. 4B) so that, in the presence of apocynin, there was no difference in myocardial superoxide release between nNOS$^{+/+}$ and nNOS$^{-/-}$ mice. Pre-incubation of nNOS$^{-/-}$ LV myocytes with ET-1 resulted in a trend towards an increase in superoxide production that did not reach statistical significance (Fig. 4B). As shown under basal conditions, apocynin greatly reduced superoxide release in nNOS$^{-/-}$ myocytes in the presence of ET-1 (Fig. 4B).

Taken together, these findings suggest that nNOS gene deletion increases the activity of myocardial NADPH oxidases and that the resulting increase in superoxide production may contribute to the greater cell shortening and LV inotropy in nNOS$^{-/-}$ mice.

Although inhibition of NADPH oxidases abolished the positive inotropic effect of ET-1 in both genotypes, we have been unable to detect a significant increase in superoxide production in myocyte lysates following incubation with ET-1. This unexpected finding may reflect the inability of our technique to detect localized superoxide release in subcellular microdomains.

3.1.3. The downstream targets of NADPH oxidase and ROS — Ca^{2+} handling proteins

The emerging role of NADPH oxidases in the myocardial response to stretch has opened the possibility that ROS formation may be routinely involved in the regulation of myocardial inotropy under physiological conditions. Indeed, several Ca^{2+} handling proteins in the mammalian myocardium are redox sensitive (*i.e.*, the L-type Ca^{2+} channel, RyR and the NCX [44]). Exogenous ROS can either increase or decrease the amplitude of L-type Ca^{2+} currents [44,45] and ET-1 has recently been shown to activate L-type Ca^{2+} channels through a mechanism that involves stimulation of NADPH oxidase activity and superoxide production in cardiac myocytes [46]. Similarly, exogenous ROS substantially enhanced NCX-mediated Ca^{2+} fluxes in cardiac sarcolemmal vesicles or in isolated cardiac myocytes [47,48]. ET-1 and PKC – both of which are known to stimulate NADPH oxidase activity [46,49] – also increase the NCX current in guinea-pig ventricular myocytes [50]. Finally, the increase in NCX activity and Ca^{2+} transient amplitude elicited by Ang II or ET-1 can be prevented by inhibiting NADPH oxidase activity [4,51,52].

Stretch-activated channels (SAC) are permeable to both Ca^{2+} and Na^{+} ions. Activation of SAC by mechanical stretch depolarizes diastolic membrane potential, prolongs action potential duration (APD) and triggers spontaneous action potentials [53]. The signaling pathways involved in the regulation of SAC by stretch are largely unknown. Phorbol 12,13-dibutyrate (PDBu), which is known to mimic diacylglycerol (DAG) and activate PKC, dose-dependently increased SAC in rat atrial myocytes. A structural analogue of DAG that does not activate PKC, 4α-phorbol, failed to show any effect on SAC. PKC inhibitor, chelerythrine (1 μM), eliminated the effect of PDBu on SAC (unpublished data). Application of oxidants activates non-selective cation channels, increase intracellular Ca^{2+} and depolarizes the membrane potential in non cardiac cells such as insulin-secreting cell line [54,55].

3.2. Myocardial signaling involved in stretch-induced inotropic responses

3.2.1. Role of protein kinase C and protein kinase D

Activation of PKC-dependent signaling in cardiac myocytes exposed to stretch or ET-1 [56] suggests that this kinase may be involved in mediating the positive inotropic response to ET-1. In cat papillary muscles or guinea-pig ventricular myocytes, PKC inhibition reversed the positive inotropic effects of ET-1 [38,57]. However, in our hands application of the PKC inhibitor, chelerythrine (CLT, 1 μM), did not affect basal cell shortening or prevent the positive inotropic effect

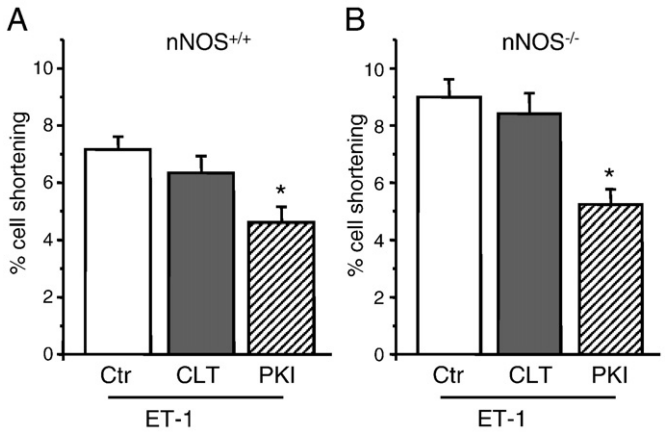

Fig. 5. Effect of PKC or PKA inhibition on the ET-1 positive inotropic response. A and B. PKC inhibition with chelerythrine (CLT, 1 μM) did not affect the inotropic effect of ET-1 in either group. In contrast, PKA inhibition with PKI (2 μM) significantly reduced the effect of ET-1 in nNOS$^{+/+}$ and nNOS$^{-/-}$ myocytes. Under basal conditions, cell shortening before and after CLT was 5.4 ± 0.4 and 5.3 ± 0.4 in nNOS$^{+/+}$, $n = 11$; 6.8 ± 0.7 and 6.6 ± 0.6 in nNOS$^{-/-}$, $n = 7$. Similarly, before and after PKI was 4.6 ± 0.3 and 3.5 ± 0.3 in nNOS$^{+/+}$, $n = 12$; 6.1 ± 0.6 and 4.4 ± 0.5 in nNOS$^{-/-}$, $n = 16$.

of ET-1 in either nNOS$^{+/+}$ or nNOS$^{-/-}$ LV myocytes (Fig. 5A and B). In the presence of CLT, ET-1 increased cell shortening from 5.3 ± 0.4% to 6.3 ± 0.6% in nNOS$^{+/+}$ myocytes ($P<0.01$, $n = 11$) and from 6.6 ± 0.6 to 8.4 ± 0.7% in nNOS$^{-/-}$ myocytes ($P<0.01$, $n = 7$). These data suggest that PKC is not involved in the positive inotropic effect of ET-1 in murine LV myocytes.

Protein kinase D (PKD) is also activated by ET-1 in cardiac myocytes mostly *via* a PKC-dependent mechanism [58]. PKD appears to target the Ser^{22} and Ser^{23} phosphorylation sites in cardiac troponin I (which are also phosphorylated by PKA) and to reduce the myofilament Ca^{2+} sensitivity [59]. This effect would be expected to result in a reduction in myocardial inotropy; however, the relevance of PKD in the fast and slow inotropic response to stretch has yet to be investigated.

3.2.2. Role of protein kinase A

Cyclic AMP-dependent signaling may play an important role in mediating the Anrep effect. For instance, Calaghan et al. [60] showed that the myocardial cAMP content was significantly increased in ferret papillary muscles that developed a slow positive inotropic response to stretch but not in those where stretch could not elicit an increase in contraction. Similarly, increased myocardial cAMP has been observed following stretch of isolated frog ventricles [61], in LV pressure-overloaded Langendorff-perfused rat hearts [62], and in the canine heart in situ following LV stretch [63]. In view of these findings, we investigated whether PKA-dependent signaling was required for the development of a positive inotropic response to ET-1 in LV myocytes from nNOS$^{+/+}$ and nNOS$^{-/-}$ mice.

As shown in Fig. 5A and B, inhibition of PKA by PKI (2 μM) significantly reduced the positive inotropic effect of ET-1 in both nNOS$^{+/+}$ and nNOS$^{-/-}$ LV myocytes. Both basal and ET-1-stimulated cell shortening was not different between nNOS$^{-/-}$ and nNOS$^{+/+}$ myocytes in the presence of PKI. These data suggest that PKA may play an important role in mediating ET-1 and stretch-mediated inotropic responses in the murine LV myocardium.

PKA activation by ET-1may involve local production of ROS from NADPH oxidase. Indeed Brennan et al. have shown that hydrogen peroxide can directly activate type I PKA by inducing the formation of an interprotein disulfide bond between its two regulatory subunits [64]. This process leads to an increase in cell shortening *via* activation and subcellular translocation of PKA, in the absence of adrenergic receptor stimulation.

Taken together, these findings suggest that activation of NADPH oxidase may lead to increased myocardial inotropy through stimulation and subcellular targeting of PKA signaling by ROS.

3.2.3. Myosin light chain kinase

Kockskämper et al. have recently suggested that MLCK may be involved in the positive inotropic effect of stretch in the human atrial and ventricular myocardium. In this work, stretch led to a significant increase in MLCK-dependent myosin light chain 2 (MLC2) phosphorylation and inhibition of MLCK blunted the stretch-induced positive inotropic effect [40]. These results are consistent with *in vivo* investigations showing that exercise-induced elevations in LV preload are associated with increased MLC2 phosphorylation [65].

4. Conclusion

Mechanical stretch plays an important role in the modulation of myocardial inotropy and growth responses both in health and in disease states. Whereas mechanical stimulation and increased LV preload have been shown to stimulate NO release from the vascular endothelium [7], which – in turn – facilitates the Frank–Starling response [9], the role of myocardial constitutive NO production in mediating either the rapid or the slow inotropic response to stretch remains controversial. It is generally accepted that the myocardial release of Ang II and ET-1 in response to stretch is instrumental in mediating the slow inotropic response or Anrep effect. In agreement with these findings, recent evidence suggests that stimulation of NADPH oxidases (a known target of Ang II and ET-1) and ROS formation may be of critical importance in mediating the Anrep effect. Our findings indicate that the positive inotropic effect of ET-1 requires intact NADPH oxidase activity and PKA signaling. The latter may be directly activated by ROS formation [64], opening the possibility that the NO-redox state of the myocardium may directly affect protein phosphorylation and the inotropic state, independent of adrenergic receptor stimulation.

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