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Early signals after stretch leading to cardiac hypertrophy. Key role of NHE-1

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1. ABSTRACT

The enhanced activity of the cardiac Na^+/H^+ exchanger (NHE-1) after myocardial stretch is considered a key step of the intracellular signaling pathway leading to the slow force response to stretch as well as an early signal for the development of cardiac hypertrophy. We propose that the chain of events triggered by stretch begins with the release of small amounts of Angiotensin II (Ang II)/endothelin (ET) and ends with the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the Na^+/Ca^{2+} exchanger in reverse mode (NCX_{rev}), which triggers cardiac hypertrophy by activation of widely recognized Ca^{2+}-dependent intracellular signaling pathways.

2. INTRODUCTION

During the last decade a substantial part of our own studies were addressed to identify the intracellular signaling triggered by myocardial stretch. In this regard, we have provided convincing evidence that myocardial stretch elicits an autocrine/paracrine loop that involves a sequential release of preformed Ang II and ET, and ends with an increase in contractility induced by Ca^{2+} influx (1-6). Furthermore, we have proposed that an enhanced activation of NHE-1 is a key factor in this signaling pathway by increasing intracellular Na^+ concentration ($[Na^+]_i$), which drives the NCX_{rev} thus increasing Ca^{2+} transient and contractility. The question as to why this mechanism should be connected to the development of myocardial hypertrophy appears to be answered by the fact that an enhanced activity of NHE-1 is detected in several models of cardiac hypertrophy where specific NHE-1 blockade regresses cardiac hypertrophy effectively (7-38).

This review will present previously published data by our own and other laboratories supporting the notion that early intracellular signals triggered after myocardial stretch may conceivably lead to cardiac hypertrophy.

3. STRETCHING ADULT MYOCARDIUM

In 1998 Bluhm *et al.* (39) published the results obtained with an elegant theoretical ionic model of

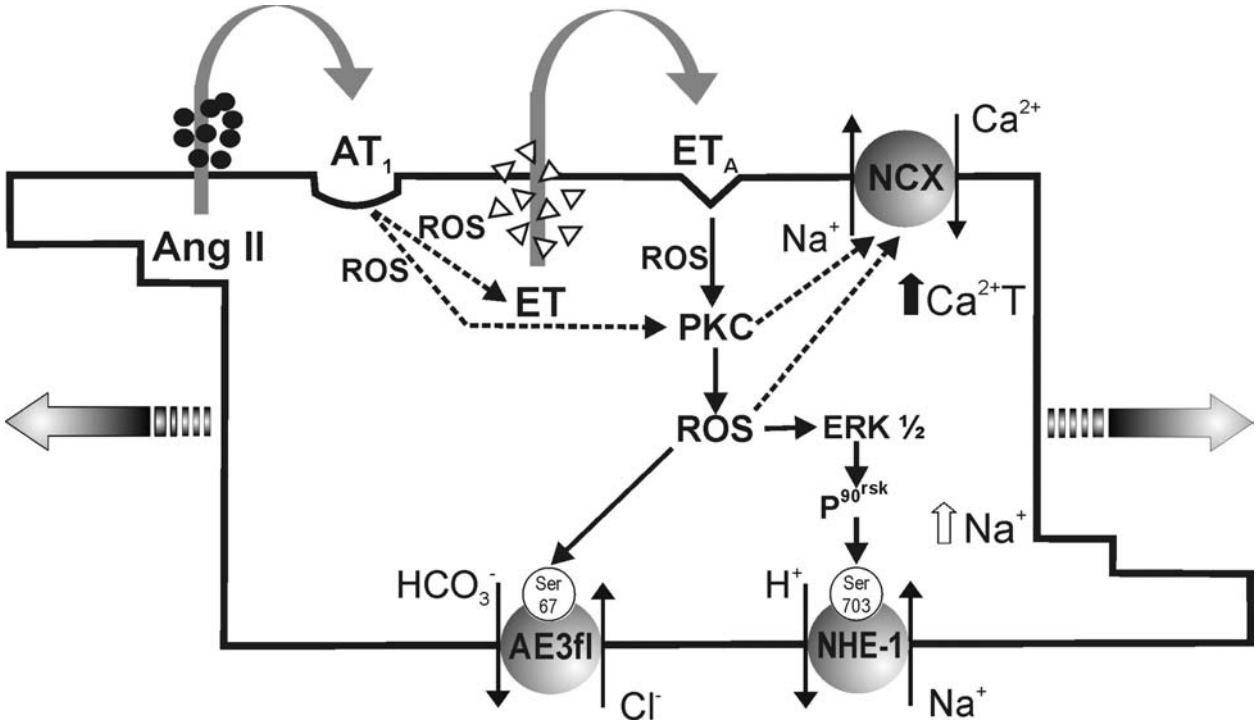

Figure 1. A representation of the proposed autocrine/paracrine cascade of events following myocardial stretch. Endogenous Ang II is released from the myocytes activating AT_1 receptors in an autocrine fashion. Stimulation of AT_1 induces the release/formation of ET, which simultaneously activates NHE-1 and Cl^--HCO_3^- exchanger through ET_A receptors. The activation of Cl^--HCO_3^- exchanger prevents the expected intracellular alkalization due to NHE-1 activation but does not prevent the rise in $[Na^+]_i$. The increase in $[Na^+]_i$ drives the NCX in its reverse mode and this, together with a probable direct action on the exchanger, leads to the increase in Ca^{2+} transient (Ca^{2+}T). Modified from Perez *et al.* (3) with permission.

ventricular myocyte used to analyze the changes in sarcolemmal ion fluxes following step changes in cardiac muscle length. They suggested that a sudden increase in muscle length might induce changes in sarcolemmal Na^+ influx leading to an increase in $[Na^+]_i$ and a concomitant increase in systolic Ca^{2+} entry through the Na^+/Ca^{2+} exchanger (NCX). However, the mechanism by which the increase in $[Na^+]_i$ takes place was not proposed. Since the NHE-1 is an important entry pathway in cardiomyocytes, the possible role played by the exchanger was analyzed.

4. NHE-1 AND MYOCARDIAL STRETCH

The finding of a stretch-induced myocardial alkalization in cat papillary muscles bathed with a bicarbonate-free medium was the first piece of evidence provided by our laboratory about the main role played by NHE-1 in the myocardial response to stretch (1). The absence of bicarbonate from the medium allowed us to analyze the role of NHE-1 without the influence of bicarbonate-dependent intracellular pH (pH_i)-regulatory mechanisms. The stretch-induced myocardial alkalization was suppressed by either Ang II type 1 (AT_1) or ET type A (ET_A) receptors blockade, suggesting the involvement of these receptors in the stretch-induced activation of NHE-1 (1). In accordance with this, the release of Ang II after stretching cultured neonatal cardiomyocytes was initially reported by Sadoshima and co-authors (40), who showed that the addition of surrounding medium from stretched to non-stretched cardiomyocytes promoted hypertrophy, and that Ang II was the autocrine/paracrine mediator of this effect. Contemporarily, Ito *et al.* (41) found in the same preparation that Ang II promotes the release/formation of ET-1, demonstrating that ET-1 is an autocrine/paracrine factor in the mechanism of Ang II-induced cardiac hypertrophy. In addition, Yamazaki *et al.* (7) found that stretch induced a rise in the concentration of ET-1 in the culture medium, where it is constitutively secreted from cardiomyocytes together with an increase in NHE-1 activity. The same authors showed that NHE-1 inhibition partially attenuated the stretch-induced mitogen-activated protein kinase activation. Therefore, our main contribution was to demonstrate the existence of a stretch-triggered autocrine/paracrine release of Ang II/ET leading to NHE-1 activation in an adult cardiac multicellular preparation (1-3). This finding allowed us to propose the hypothetical scheme depicted in Figure 1. The proposed chain of events begins with the release of preformed Ang II and ends with an increase in the Ca^{2+} transient through NCX_{rev} activation secondary to the NHE-1 activation-mediated rise in $[Na^+]_i$. If we analyze the potential effects of NHE-1 activation on myocardial contractility, we should consider two different mechanisms: Na^+-triggered increase in the Ca^{2+} transient through NCX, and an increase in pH_i that would increase the contractile force by increasing myofilament Ca^{2+} responsiveness. Considering the latter possibility, it is

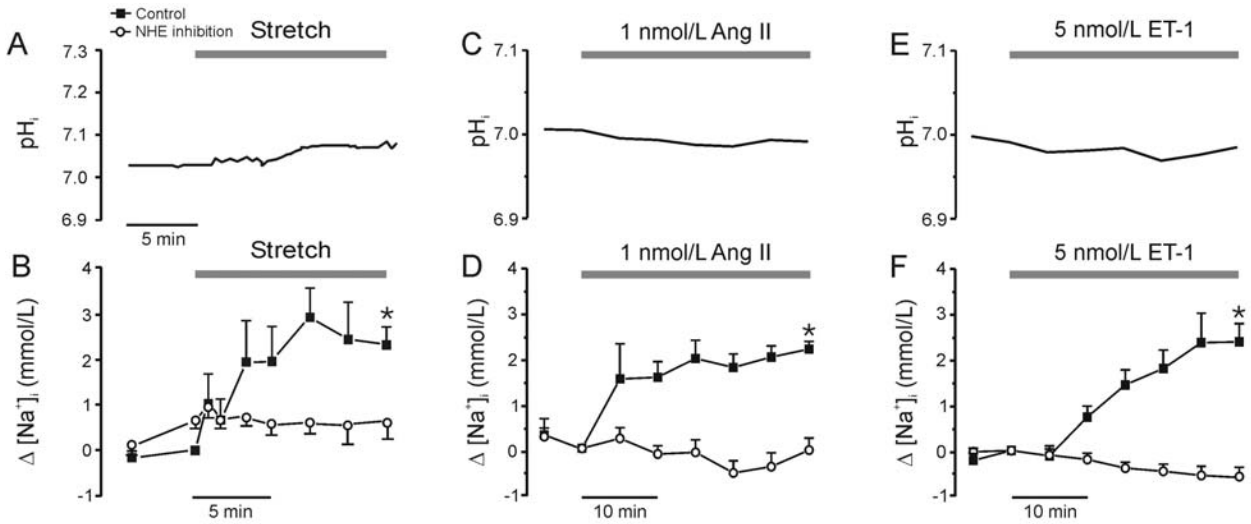

Figure 2. Representative experiments showing that in the presence of bicarbonate**,** NHE-1 activation by stretch (Panel A), exogenous Ang II (Panel C) or ET-1 (Panel E) does not change pH_i. The increase in $[Na^+]_i$ observed under each condition was prevented by NHE-1 blockade (pooled results of Panels B, D and F)**.** * Indicates $P<0.05$ vs. NHE inhibition. Modified from Perez *et al.* (42) with permission.

important to emphasize that little or no change in pH_i is detected when the stimulating effect of stretch, exogenous Ang II or ET-1 on NHE-1 is studied in the presence of bicarbonate buffers (1-3,42-44). The explanation for the lack of change in pH_i can be found in the fact that growth factors like Ang II and ET simultaneously activate at least two opposing pH_i-regulatory mechanisms: the alkalinizing NHE-1 and the acidifying Na^+-independent Cl^--HCO_3^- anion exchanger (4,42,45-49). The scheme in Figure 1 illustrates the fact that Ang II -through release/formation of ET-, simultaneously stimulates NHE-1 and Cl^--HCO_3^- exchanger, thus minimizing the changes in pH_i but without affecting the increase in $[Na^+]_i$ that follows NHE-1 activation. Therefore, NHE-1 activation can be detected as a pH_i increase only if bicarbonate is absent from the medium. We emphasize this point because the absence of changes in pH_i after growth factor stimulation in bicarbonate media is not widely recognized, though it was reported by Ganz *et al.* (47) in 1988 in mesangial cells and a call for attention was published by Thomas (48) in a letter to Nature one year later. More recently, Shafer *et al.* (8) demonstrated that the hypertrophic response of cardiomyocytes to α- and β-adrenergic stimulation requires NHE-1 activation but not cellular alkalization. In summary, although there is much evidence to suggest a direct correlation between activation of cellular acid extrusion mechanisms and proliferation, there is enough evidence to suggest that proliferation can occur without changes in pH_i, and that changes in pH_i do not necessarily induce proliferation (8,47,50,51). In human fibroblasts, bradykinin activates NHE-1 but does not influence cell proliferation (52). Regarding growth and viability in NHE-deficient mutants, there is no agreement in the literature. While some authors report that these animals can grow at normal rate (53), others have shown that NHE-$1^{-/-}$ mice exhibit growth retardation and are subject to slow-wave epilepsy (54-56).

The effects of myocardial stretch, exogenous Ang II and ET-1 on pH_i and $[Na^+]_i$ in cat papillary muscles are illustrated in Figure 2. In these experiments, exogenous Ang II or ET-1 at low doses that probably reproduced those released after stretch did not affect pH_i but significantly increased $[Na^+]_i$. This rise in $[Na^+]_i$ was suppressed by NHE-1 inhibition. The ET receptor blockade had the same inhibitory effect after myocardial stretch and after addition of exogenous Ang II or ET-1. The role played by the Cl^--HCO_3^- exchanger in preventing intracellular alkalization after myocardial stretch is better visualized by repeating the intervention in a bicarbonate medium before and after inhibition of the anion-exchanger with specific antibodies (see Figure 3) (4). Under these conditions, an increase in pH_i takes place only after Cl^--HCO_3^- exchanger inhibition. Whether changes in pH_i after the addition of growth factors or stretch stimulation localized to certain subcellular spaces within the myocyte may occur in the presence of bicarbonate-dependent mechanisms is not clear. The fact that an increase in pH_i stimulates protein synthesis (57) does not necessarily mean that intracellular alkalization occurs after myocardial stretch, Ang II or ET-1 stimulation (5,8,47).

It is known that the increase in $[Na^+]_i$ can induce an increase in $[Ca^{2+}]_i$ through the NCX as a result of a decrease in Ca^{2+} efflux (decreased forward mode) and/or an increase in Ca^{2+} entry (increased reverse mode). As mentioned before, the increase in $[Na^+]_i$ induced by stretch or by exogenous low doses of Ang II or ET-1 was prevented by blocking NHE-1 (Figure 2) (2,3,42,44). The increase in myocardial $[Na^+]_i$ detected in our experiments was ~3–6 mmol/L. In line with this, increases of similar magnitude were detected by Baartscheer *et al.* (9) in the myocardium of rabbit failing hearts with enhanced activity of NHE-1 and by Luers *et al.* (43) after stretching rabbit myocardium. This increase in $[Na^+]_i$, shifts the reversal potential of NCX to a more negative voltage, giving more time for NCX to operate in reverse mode during the action potential and promoting Ca^{2+} influx to the cell which should be reflected

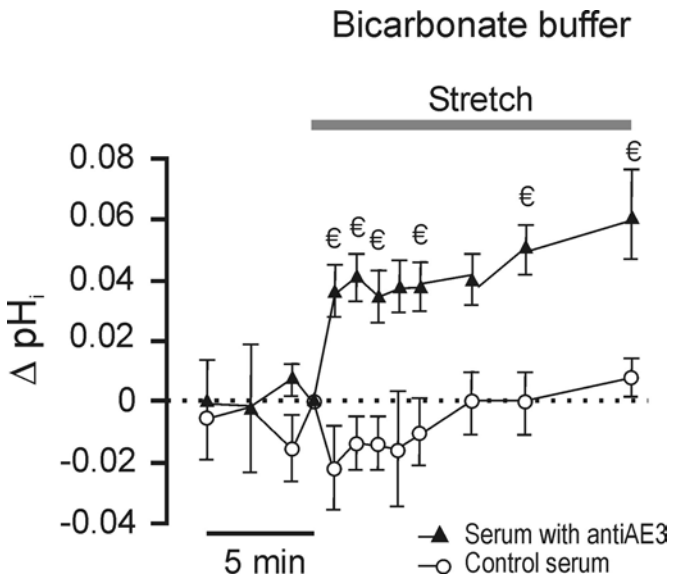

Figure 3. When Cl^--HCO_3^- exchanger activity is inhibited by a specific antibody against it, slow increase in force after stretch is even greater than when the anion exchanger is operative, due to a rise in pH_i despite the presence of extracellular bicarbonate. Under this condition, the increase in myofilament responsiveness increases developed force in addition to the effect of the augmented Ca^{2+} transient. € P <0.05 vs. control serum. Modified from Cingolani *et al.* (4) with permission.

by changes in contractility. As reported by Bers *et al.* (58), cardiomyocytes have a limited capacity to buffer increases in $[Na^+]_i$ and NCX is more sensitive than the Na^+/K^+ ATPase pump to a change in $[Na^+]_i$ of this magnitude.

Calculation of the estimated reversal potential of NCX in cat papillary muscles gives a value of -34 mV which is of the same order of magnitude as those estimated by other authors (59,60), if we assume 10 mmol/L $[Na^+]_i$, 140 mmol/L extracellular (Na^+), 1.5 mmol/L extracellular Ca^{2+} and a 150 nmol/L diastolic $[Ca^{2+}]_i$. The quick rise in sub-membrane $[Ca^{2+}]_i$ due to the Ca^{2+} transient that shifts the NCX reversal potential to even more positive voltages (61) would lead to a minimal contribution of the NCX_{rev} to basal contractility under normal conditions (3,42,44). In accordance, we have shown that NCX_{rev} inhibition with KB-R7943 did not affect basal contractility or an increase in contractility of ~20% promoted by rising extracellular Ca^{2+} from 1.35 mmol/L to 1.9 mmol/L (Figure 4) in cat myocardium (42). However, these results are in contrast to those obtained by Kurogouchi *et al.* (62) in the dog myocardium who showed that KB-R7943 promoted a pronounced negative inotropic effect, discrepancy that might depend on the model and/or species used in each study. The approximately 3-6 mmol/L increase in $[Na^+]_i$ induced by stretch (34), exogenous Ang II (42) or ET-1 (44) in our experimental conditions certainly changes the scenario by shifting the reversal potential of NCX from -34 to -55 mV, allowing operation of the NCX reverse mode during a longer fraction of the action potential plateau. In line with the above-mentioned effect of stretch, Ang II and ET on $[Na^+]_i$, we detected a negative shift of the NCX reversal potential of -5 mV and -15 mV after treating isolated patch-clamped cat myocytes with 1 nmol/L and 10 nmol/L ET-1, respectively (44). Considering these experimental results, estimation of the ET-1-induced increase in $[Na^+]_i$ gives values of approximately 1.6 mmol/L and 5.0 mmol/L for 1 nmol/L and 10 nmol/L ET-1, respectively. These values are of the same order of magnitude as those measured in the bulk of the cytosol by epifluorescence in papillary muscles after addition of 5 nmol/L ET-1 (42). However, it is important to note that the increase in $[Na^+]_i$ in the isolated myocytes might reflect changes of this ion in a space in which intracellular dialysis with the solution of the patch pipette cannot maintain $[Na^+]$ at a constant level. The increase in $[Na^+]_i$ would tend to increase Ca^{2+} influx through reverse mode NCX during systole and to reduce Ca^{2+} extrusion via forward mode NCX during diastole that should necessarily end with an increase in force of contraction as reported by us (2,3,42,44).

We have reported an increase in the Ca^{2+} transient of about 12 % during the slow force response that was due to an increase in its amplitude without changes in diastolic Ca^{2+} (2,3), result that coincides with that reported by Kentish and Wrzosek (63). These findings suggest a cell Ca^{2+} influx from the extracellular space during the slow force response, supporting the notion that reverse NCX activation would be responsible of the increase in Ca^{2+} transient. The reported lack of participation of the sarcoplasmic reticulum in this mechanism (63-65) further supports this idea.

The question that now arises is if this increase in $[Ca^{2+}]_i$ secondary to the increase in $[Na^+]_i$ is the only mechanism responsible for the positive inotropic effect when Ang II or ET is involved in the mechanism? Figure 5 shows that developed force increases linearly with the increase in $[Na^+]_i$ caused by Na^+/K^+-ATPase inhibition, and that this increase is blunted by KB-R7943 (Figure (5 inset)). However, when $[Na^+]_i$ increases because of ET-1-induced activation of NHE-1 (44), the increase in developed force

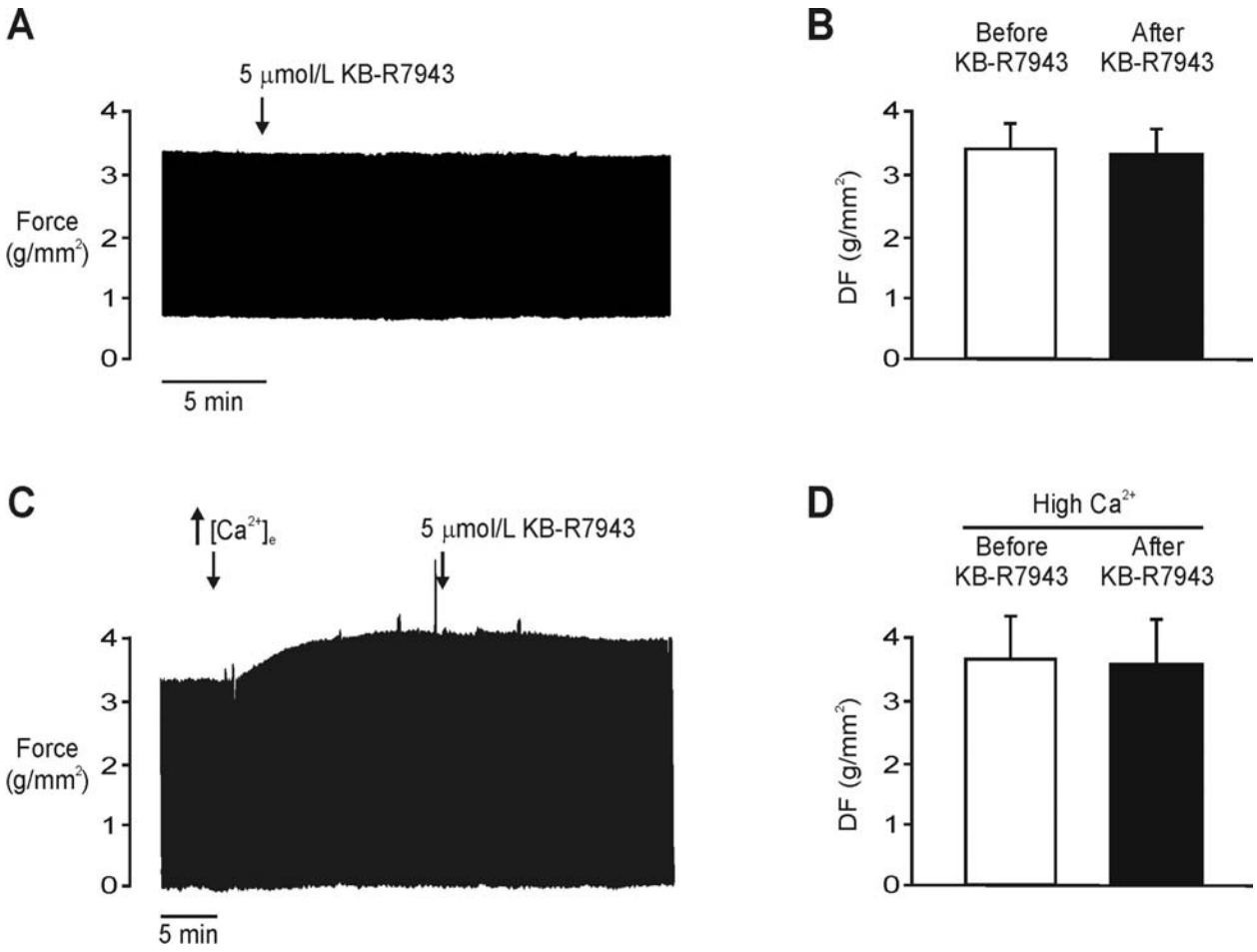

Figure 4. Original force records showing the lack of effect of 5 μmol/L KB-R7943 (NCX_{rev} blocker) on basal contractility (A, extracellular Ca^{2+} = 1.35 mmol/L) and on the increase in contractility of ~20% promoted by increasing extracellular Ca^{2+} from 1.35 mmol/L to 1.9 mmol/L (C). Overall results of developed force (DF, in g/mm^2) for each type of experiments (B, n=6 and D, n=4). These results also suggest strongly that KB-R7943 at this concentration does not exert non-specific actions which may affect contractility. Reproduced from Perez *et al.* (42) with permission.

lies above the linear relationship (Figure 5). In addition, if ET-1 is applied when the rise in $[Na^+]_i$ caused by Na^+/K^+-ATPase inhibition reached a steady state in the presence of NHE-1 inhibition, the peptide produces a positive inotropic effect that is completely reversed by either inhibition of NCX_{rev} or protein kinase C (44). Patch-clamp experiments in isolated myocytes showed that ET-1 increases the NCX current and negatively shifts the NCX reversal potential (44). Taken together, these data suggest that ET-1 is driving the reverse mode of NCX by an NHE-1-mediated increase in $[Na^+]_i$ and by a direct stimulatory effect on the NCX, possibly by a protein kinase C-dependent phosphorylation mechanism (44).

Interestingly, experiments performed by Eigel *et al.* (66) in guinea pig ventricular myocytes demonstrated that reactive oxygen species (ROS) activate NCX directly. On the other hand, it was reported that Ang II or myocardial stretch, via AT_1 receptors stimulation, induces a reactive oxygen species-mediated reduction of the transient outward potassium current by a signaling pathway involving NADPH oxidase activation (67). Thus, decreased transient outward potassium current would lead to a prolongation of action potential duration, which may eventually increase Ca^{2+} influx through NCX_{rev}.

In summary, it may be suggested that the reverse mode of cardiac NCX is modulated by myocardial stretch or, equivalently, by the Ang II/ET network, through three different pathways: a) an $[Na^+]_i$-dependent pathway, consistent with a negative shift of the NCX reversal potential after a rise in $[Na^+]_i$ due to NHE-1 activation; b) an $[Na^+]_i$-independent and protein kinase C-dependent pathway by direct stimulation of NCX; and c) a prolongation of the action potential duration. All of them appear to be contributing in concert after stretch. However, the $[Na^+]_i$-independent pathway seems to contribute to the mechanism only after the primary participation of the $[Na^+]_i$-dependent pathway, which appears to be a mandatory step (44).

The fact that Ang II triggers the beginning of the cascade of events leading to the show force response has

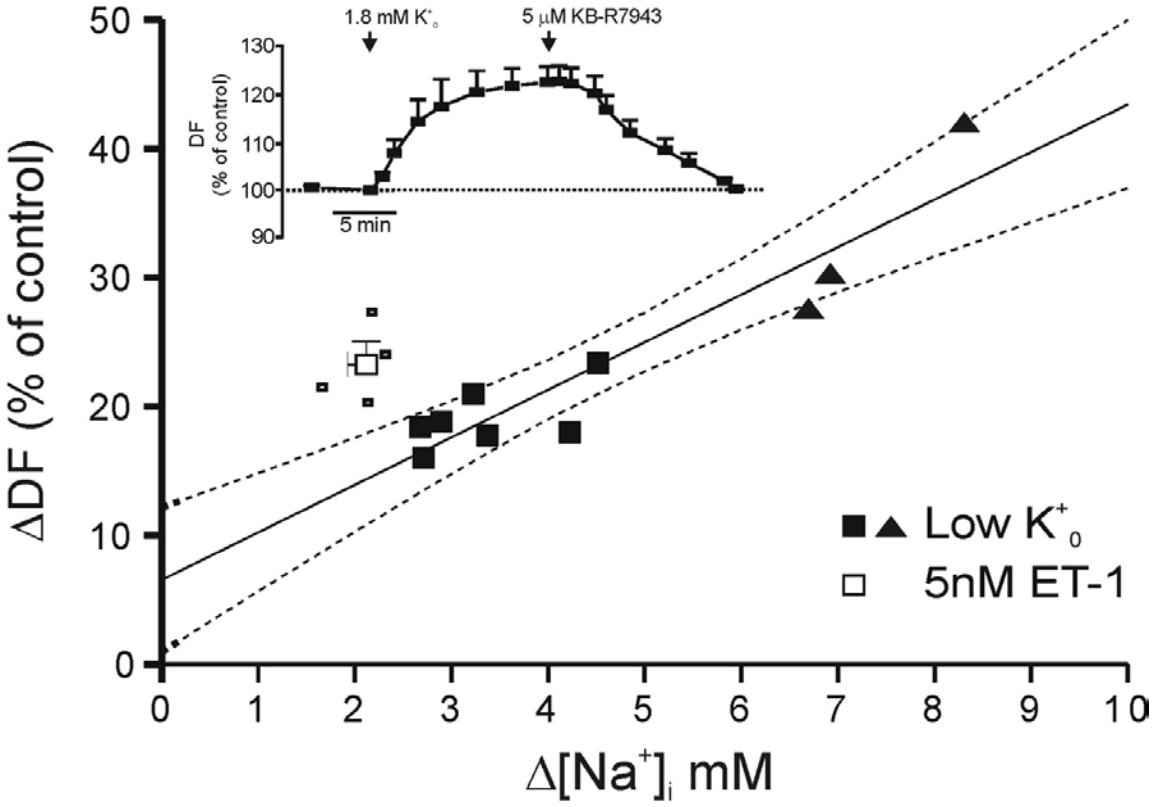

Figure 5. The increase in $[Na^+]_i$ induced by partial inhibition of Na^+/K^+ ATPase by lowering extracellular K^+ increased DF as a function of $[Na^+]_i$. This effect may be assigned to activation of NCX_{rev}, because it was reverted by KB-R7943 (5 μmol/L; inset). However, when $[Na^+]_i$ levels were augmented by ET-1–induced NHE activation, the results lied above the relationship, suggesting that factors additional to the rise in $[Na^+]_i$ have taken place. Modified from Aiello *et al.* (44) with permission.

not been confirmed by many authors in all their steps. Activation of the NHE-1 after stretch has been confirmed for different species by several authors (2,10,43,68,69). However, the pathway leading to its activation is controversial. The release of Ang II and activation of the AT_1 receptors by stretch proposed by us in rat and cat (1-3), though reported in isolated rat myocytes (40,70), was not confirmed by other investigators in ferret multicellular preparations (71). The role played by ET, the second step in the chain of events, has been reported by Calaghan and White in ferret (71) and by us in rat (2) and cat (1,3), but was not found by von Lewinski *et al.* in rabbit (72) or failing human myocardium (69). Whether the discrepancies are a matter of species differences is not apparent to us yet, but in any case, they leave open the possibility that under different experimental conditions some other mechanisms may be triggered after stretch. In this regard, other report by Calaghan and White (68) shows activation of stretch-activated channels in addition to the NHE-1 after myocardial stretch; Isenberg *et al.* (73) proposed that myocardial stretch increases $[Na^+]_i$ and $[Ca^{2+}]_i$ in cell organelles partly by their influx through the stretch-activated channels, while they were unable to prevent the increase in $[Na^+]_i$ by cariporide. Vila Petroff *et al.* (74) presented evidence that stretch activates the PI-3-kinase pathway to phosphorylate the endothelial isoform of nitric oxide synthase. Then nitric oxide stimulates Ca^{2+} release from the sarcoplasmic reticulum and promotes the slow force response. Unfortunately, the results of Vila-Petroff *et al.* could not be reproduced by other authors either in papillary muscle or isolated myocytes (68). This is expected since this mechanism requires a functional sarcoplasmic reticulum and the possible role of the sarcoplasmic reticulum in the slow force response has been ruled out by several authors including Bluhm and Lew (64) Hongo *et al.* (65) and Kentish and Wrzosek (63).

Another important aspect to be considered to clarify the failure of detecting if ET is participating in the slow force response to stretch is to analysing the pharmacological intervention used to prove it. In this regard, Endoh *et al.* have clearly shown that high doses of the non-specific ET receptor antagonist TAK044 were necessary to prevent the inotropic effect of ET in the myocardium (75). In our hands, either TAK044 or the selective ETA receptor antagonist BQ123 blunted the slow force response (2,3). However, if based on the works of Calaghan and White (71) and our own results (1-3) the role of ET after stretch is accepted in addition to the well known fact that Ang II induces release/formation of ET as shown in different studies by us (42,45,76,77) and others (41,78-89), the rationale to accept our proposed chain of events seems to be plausible.

Regarding the identification of the ET isoform (s) that could be participating after stretch, experiments in cat

papillary muscles from our own laboratory demonstrated an increase in ET-3 mRNA after stretch (6). However, we should bear in mind that Tamamori *et al.* (90) presented evidence, at least in cultured neonatal cardiomyocytes, that ET-3 triggers the synthesis and release of ET-1 that probably mediates hypertrophic response. Therefore, though speculative, we should consider the possibility that the stretch of multicellular preparations triggers ET-3 release, which in turn increases the release/formation of ET-1. This complex mechanism and species-dependent differences may explain the discrepancies found in the signaling pathway leading to NHE-1 activation by stretch.

We can state that myocardial stretch-induced NHE-1 activation and the role of NCX in increasing Ca^{2+} transient are confirmed facts. Considering the results of different investigators and ours (1-3,5,43,68) together with those from the experiments in isolated neonatal cardiomyocytes (10), we can conclude that NHE-1 activation induced by myocardial stretch constitutes a relevant intracellular signal. This signaling pathway can be also evoked by equipotent doses of exogenous Ang II or ET-1 (42).

5. THE SLOW FORCE RESPONSE IS THE MECHANICAL COUNTERPART OF THE AUTOCRINE/PARACRINE MECHANISM TRIGGERED BY STRETCH AND MAY EXPLAIN THE ANREP's PHENOMENON

It is well known that two consecutive phases characterize the increase in force after myocardial stretch: One immediate and the slow force response. The initial rapid change in force is induced by an increase in myofilament Ca^{2+} responsiveness without changes in the Ca^{2+} transient whose underlying mechanisms are beyond the scope of this review. The slow force response, in turn, is due to a progressive increase in the Ca^{2+} transient without changes in myofilament Ca^{2+} responsiveness (2,63,91) that appears to result from the autocrine/paracrine mechanism described in the previous section. While the initial change in force after stretch seems to express Frank-Starling mechanism, the slow force response may conceivably be the expression of Anrep's phenomenon.

In 1912, Von Anrep (92) observed that when aortic pressure was elevated, ventricular volume initially increased and then declined to the starting volume. It appeared to him that an influence operating soon after myocardial dilatation caused an increase in myocardial contractility. His interpretation was that perhaps, the decrease in the flow to the adrenal glands induced the release of catecholamines and the consequent positive inotropic effect. In 1959, experiments by Rosenblueth *et al.* (93) indicated that an increase in coronary perfusion pressure was not necessarily concomitant with the return of the heart to its initial volume. In 1960, Sarnoff *et al.* (94) coined the term "pressure-induced homeometric autoregulation" to define the decrease in left ventricular end diastolic volume that follows an increase in diastolic volume due to a sudden increase in afterload. On the other hand, since the experiments of Sarnoff *et al.* (94) were performed in isolated hearts, the study served to rule out the possibility of a role played by catecholamines in the described phenomenon. Interestingly, Sarnoff defined as "homeometric autoregulation" a phenomenon occurring in an organ which was not attributable to an influence by nerves or chemicals in its vicinity, paving the way for the idea of an autocrine/paracrine mechanism after cardiac stretching (94). The existence of a real change in contractility during the homeometric autoregulation was challenged by the possibility of changes in coronary blood flow distribution (95). However, in 1973 Parmley and Chuck (96) reproduced for the first time the contractile effect of stretch in isolated strips of ventricular myocardium. They showed that when the length of the muscle was increased, there were corresponding rapid and slow increases in the developed force. Since the slow force response to the change in length was still present in isolated muscles from animals treated with reserpine, those authors also ruled out the possibility of catecholamines released by nerve endings as having a role in the mechanism.

We and other authors have provided evidence that activation of NHE-1 after stretch participates in the development of slow force response (2,3,43,68,69), however, there is no agreement in that the release of preformed Ang II mediates this activation (2,3,40,70,71). Ang II is an octapeptide acting through its own G coupled receptors AT1 and AT2. $G\alpha q\beta\gamma$ activated by either Ang II or ET-1 targets the NHE through extracellular signal-regulated protein kinases 1/2 (ERK1/2)-p90 ribosomal S6 kinase (p90rsk). We showed that the slow force response was abolished by AT1 receptors blockade (2,3,97) but not by AT2 receptors blockade (97) as shown in Figure 6A. These results support the notion that Ang II is released after stretch and triggers the intracellular signaling pathways leading to slow force response. Furthermore, a significant increase in ERK1/2 and p90rsk kinases phosphorylation can be detected after 15 minutes of stretch, effects that are both cancelled by AT1 receptors blockade with losartan as shown in Figure 6B (97). Finally, inhibition of MEK (a kinase that is upstream of ERK1/2 and downstream of RAS kinases) by PD98059 abolished slow force response to stretch (Figure 6C).

6. ROLE OF ROS AFTER STRETCH

Ang II and ET-1 are well known activators of the Nicotinamide-Adenine Dinucleotide Phosphate (NADPH) oxidase (98-100) and through this action it has been reported the phenomenon called "ROS-induced ROS-release", by which a small amount of ROS activates a greater ROS production from the mitochondria (101,102). The possibility that this mechanism may be acting in the chain of events following stretch was examined. Figure 7A shows that stretch -in addition to its mechanical effect- induced an increase in intracellular ROS formation of approximately 30% above baseline levels. Furthermore, scavenging of ROS by MPG or EUK8 inhibited both stretch-induced increase in ROS (Figure 7A) and slow force response (Figure 7B). We also found that the scavenging of ROS inhibited the increase in $(Na^{+})_i$ that occurs in response to the stretch (Figure 7C).We may

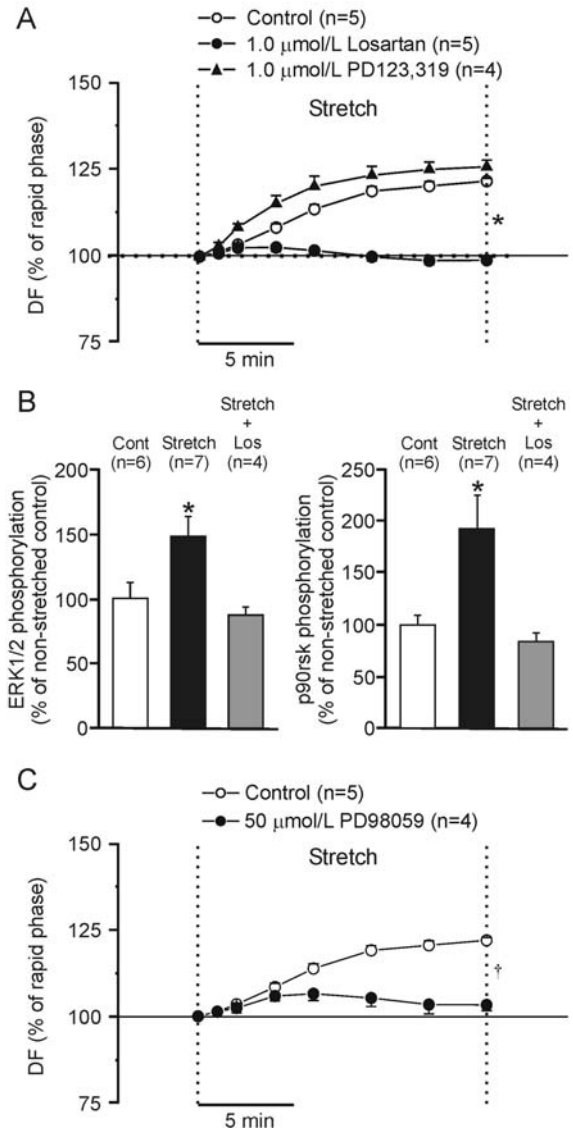

Figure 6. Suppression of the slow force response (expressed as percent of initial rapid phase) after AT1 but not AT2 receptors blockade (Losartan and PD123,319 respectively) (Panel A). Myocardial stretch significantly increased ERK1/2 and p90rsk phosphorylation, effect cancelled by losartan (Los) (Panel B). Inhibition of MEK (a kinase upstream ERK1/2 and downstream RAS) by PD98059 cancelled slow force response (expressed as percent of the initial rapid phase) (Panel C). * indicates $P < 0.05$ vs. non-stretched control (cont); † indicates $P < 0.05$ control vs. PD98059. Modified from Caldiz *et al.* (97) with permission.

hypothesize that activation of NAPDH oxidase after stretch would produce a small amount of superoxide anions ($\cdot O_2^-$), which may open the ATP-sensitive mitochondrial potassium (mKATP) channels and produce a larger amount of $\cdot O_2^-$ responsible for generating the slow force response. Therefore, if these assumptions were correct, the slow force response should be abolished by either NADPH oxidase inactivation or blockade of mKATP channels. As shown in Figure 8A, slow force response was abolished after inhibition of NADPH oxidase inhibition (apocynin or diphenyleneiodonium chloride) or after blockade of mKATP channels (5-hydroxydecanoate or glybenclamide). The NHE-1-induced increase in $(Na^+)_i$ accompanying slow force response was also abolished by these interventions (Figure 8B).

When $\cdot O_2^-$ production was augmented in cat cardiac slices by Ang II, the effect was abolished by AT1 receptors blockade (losartan), ROS scavenging (N- (2-mercaptopropionyl)-glycine, MPG), NADPH oxidase inhibition (apocynin) and mKATP channels blockade (5-hydroxydecanoate or glybenclamide) as shown in Figure 9.

7. NHE-1 ACTIVATION, THE MECHANICAL EFFECT AND MYOCARDIAL HYPERTROPHY

The possible link between slow force response to stretch and myocardial hypertrophy is supported by the fact

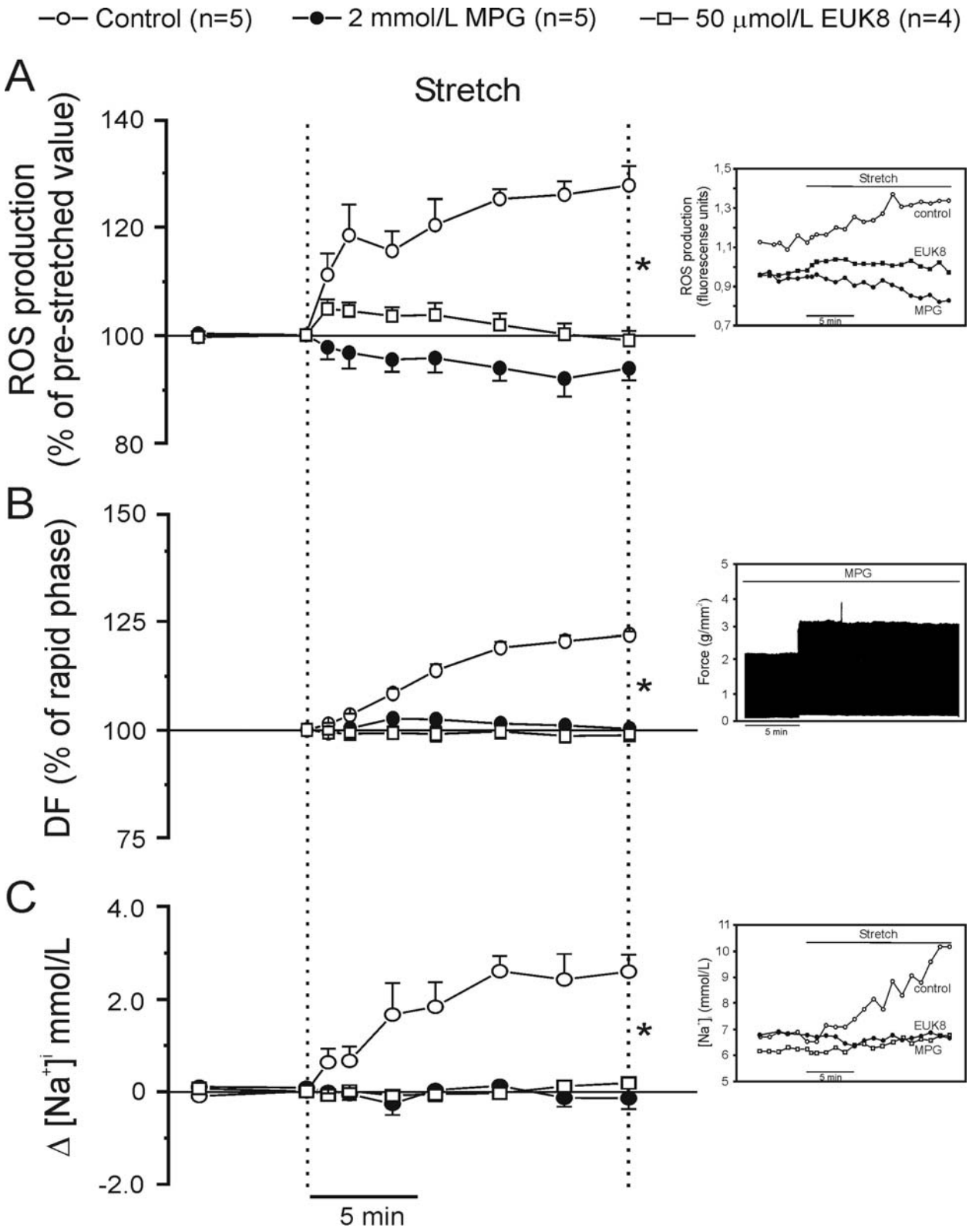

Figure 7. Myocardial stretch induced an intracellular ROS increase of ~30 % above the baseline levels that was cancelled by the ROS scavengers MPG and EUK8 (Panel A). MPG and EUK8 also cancelled the SFR (expressed as percent of initial rapid phase) (Panel B). Furthermore, ROS scavenging also blunted stretch-induced increase in $(Na^+)_i$ (Panel C). Insets show original raw data. * indicates $P < 0.05$ control vs. MPG and EUK8. Modified from Caldiz *et al.* (97) with permission.

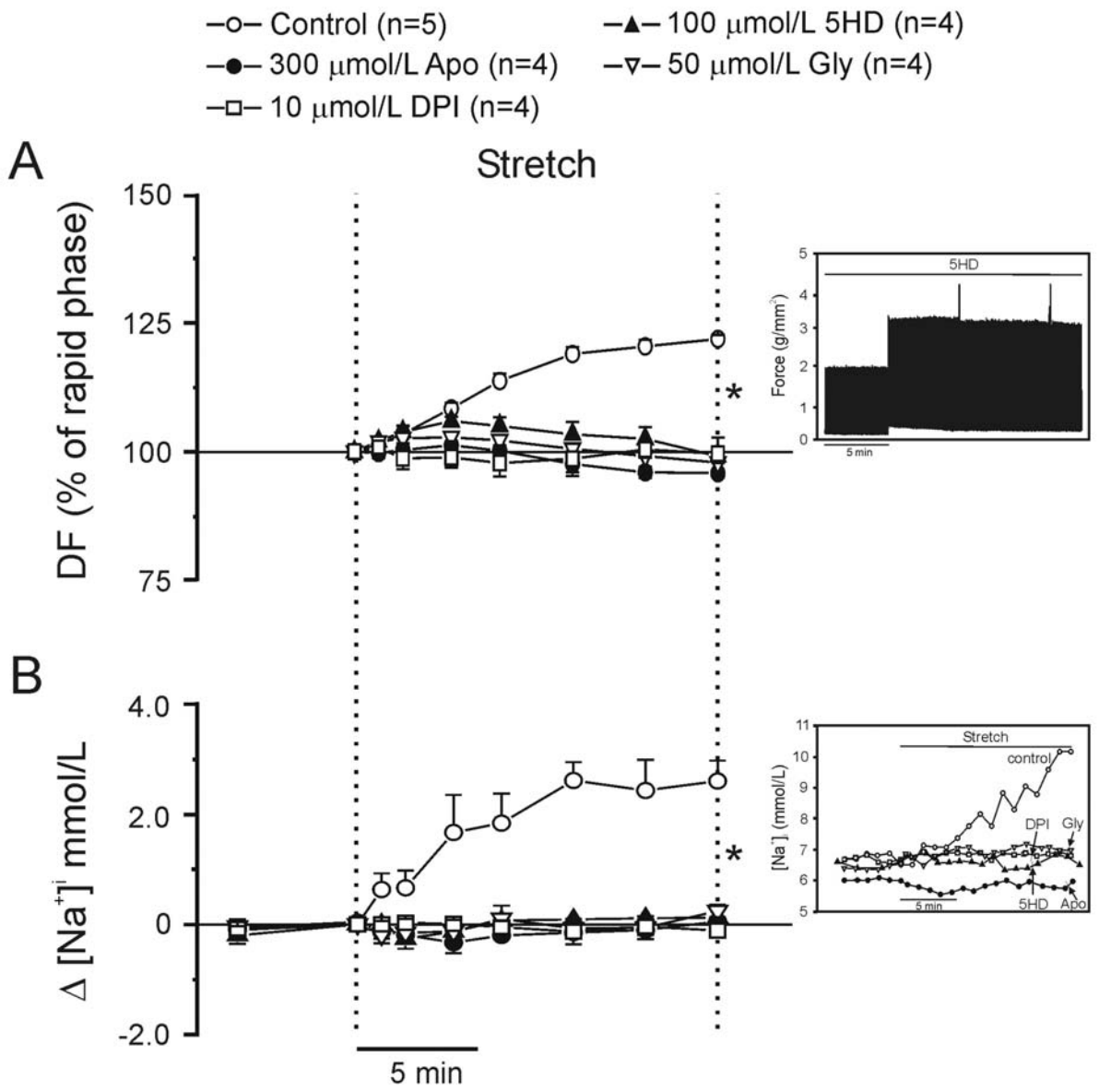

Figure 8. NADPH oxidase inhibition by Apocynin (Apo) or diphenyleneiodonium chloride (DPI) as well as mKATP channels blockade with 5-hydroxydecanoate (5HD) or glybenclamide (Gly) abolished slow force response (expressed as percent of initial rapid phase) (Panel A). All these interventions also cancelled NHE-1-mediated increase in $[Na^+]_i$ that accompanied the slow force response (Panel B). Insets show original raw data. * indicates $P < 0.05$ control vs. all other groups. Modified from Caldiz *et al.* (97) with permission.

that an enhanced activity of NHE-1 -the cause of the slow force response- is detected in several models of cardiac hypertrophy and, consistent with this, the specific blockade of NHE-1 has been shown to regress cardiac hypertrophy effectively in different models (7-38). The increase in $[Ca^{2+}]_i$ is widely recognized as one of the main prohypertrophic intracellular signals. It activates several intracellular pathways like calcineurin/ nuclear factor of activated T cells (NFAT), Ca^{2+}/calmodulin-dependent kinase II, protein kinase C and possibly other intracellular signaling pathways. Nevertheless, we emphasize that $[Ca^{2+}]_i$ may increase and induce cardiac hypertrophy by mechanisms other than those triggered by the hyperactivity of NHE-1. It has been recently suggested that Ca^{2+}/calmodulin-dependent kinase II is preferentially activated by an increase in a specific subcellular Ca^{2+} pool localized in the perinuclear area after ET-1 stimulation (103).

In 1995 enhanced activity of NHE-1 was reported in the hypertrophied myocardium of SHR (11,12). The hyperactivity of NHE-1 has been described in several tissues other than the myocardium, like in hypertensive

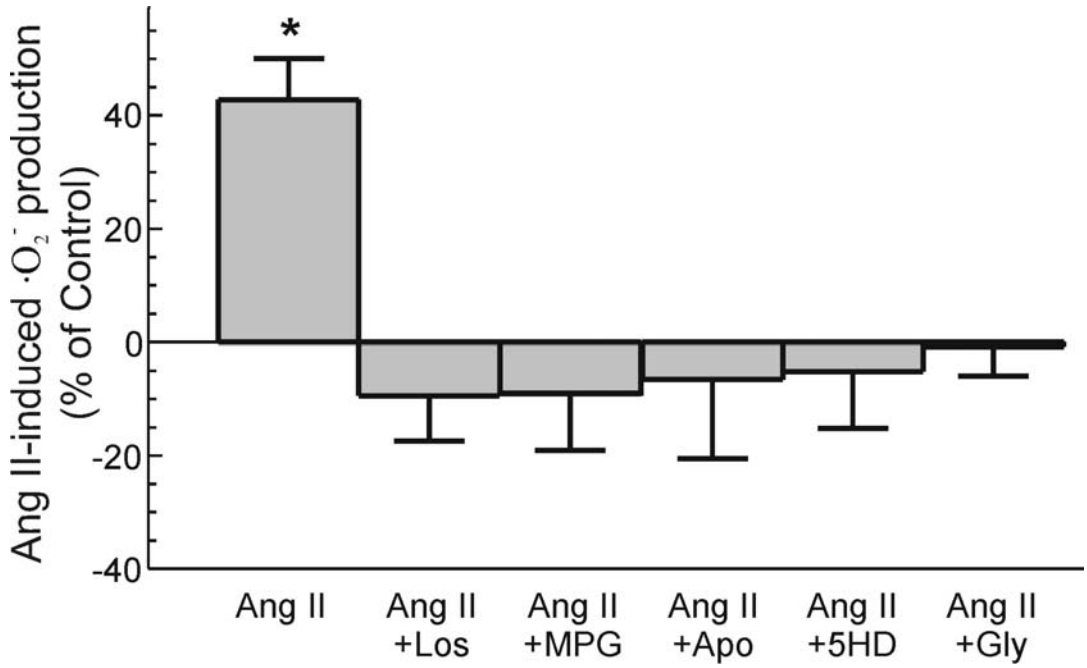

Figure 9. Superoxide production induced by 1 nmol/L Ang II (n=34) in the absence and presence of 1 μmol/L losartan (Los, n=8); 2 mmol/L N-(2-mercaptopropionyl)-glycine (MPG, n=3); 300 μmol/L apocynin (Apo, n= 7); 100 μmol/L 5-hydroxydecanoate (5HD, n=10) and 50 μmol/L glybenclamide (Gly, n=6), expressed as percent of control values without additions and after 15 minutes of incubation. * indicates $P < 0.05$ vs. control. Modified from Caldiz *et al.* (97) with permission.

humans and experimental animal models (104-106). Experiments in our laboratory showed that hyperactivity of NHE-1 in the myocardium of the SHR was not accompanied by an increase in pH_i, since there was a simultaneous activation of the acidifying Cl^--HCO_3^- exchanger (12). We also reported that the NHE-1 increased activity in this model was the result of a protein kinase C-dependent post-translational modification of the exchanger (107). It was further hypothesized that the inhibition of the antiporter activity could regress and/or prevent the development of hypertensive hypertrophy. Kusumoto *et al.* (13) proved that NHE-1 was upregulated after myocardial infarction and that the specific inhibition of this exchanger with cariporide decreased hypertrophy and remodeling in these hearts. Experiments from our own laboratory demonstrated that myocardial hypertrophy of spontaneously hypertensive rats (SHR) regressed after 1-month cariporide treatment (Figure 10) without significantly changing the arterial pressure (14). In addition, we reported that chronic NHE-1 blockade normalized the enhanced interstitial fibrosis of these hypertrophic hearts, but this effect took longer to occur compared to the regression of myocytes size (108) (Figure 11), possibly as a reflection of lower turn-over rate of collagen (109).

The precise mechanism by which NHE-1 inhibition prevents hypertrophy is still unknown, though a number of pathways have been proposed (110). As there is evidence that calcineurin plays a key role in many pathological models of cardiac hypertrophy (111-117), we recently investigated its participation in the signaling pathway involved in the regression of cardiac hypertrophy induced by NHE-1 inhibition. We analyzed the expression of β-isoform of calcineurin A (CnAβ) as an indication of calcineurin activity. The nuclear abundance of NFAT in the left ventricular myocardium of untreated SHR, treated SHR and normotensive rats was measured as a confirmation of calcineurin activation. CnAβ expression and NFAT nuclear abundance are augmented in the hypertrophied myocardium of untreated SHR, compared with the normotensive rats, and that the regression of cardiac hypertrophy induced by NHE-1 inhibition normalizes both (Figure 12) (118). An increased activity of calcineurin in the myocardium of the SHR, and its suppression by the treatment with Ca^{2+} channel blockers has been previously reported (117). This was the first report showing that the regression of cardiac hypertrophy caused by NHE-1 inhibition, which is independent from any change in blood pressure, is accompanied by normalization of CnAβ expression and NFAT nuclear abundance.

Even though we have provided evidence that a decrease in CnAβ and nuclear NFAT expression takes place during the regression of cardiac hypertrophy induced by NHE-1 inhibition, we cannot rule out the possibility of additional effects of the pharmacological interventions. It has been proposed that cariporide might exert effects at the mitochondrial level (29, 119-121).

The link between NHE-1 activity and myocardial growth has been established for several neurohumoral

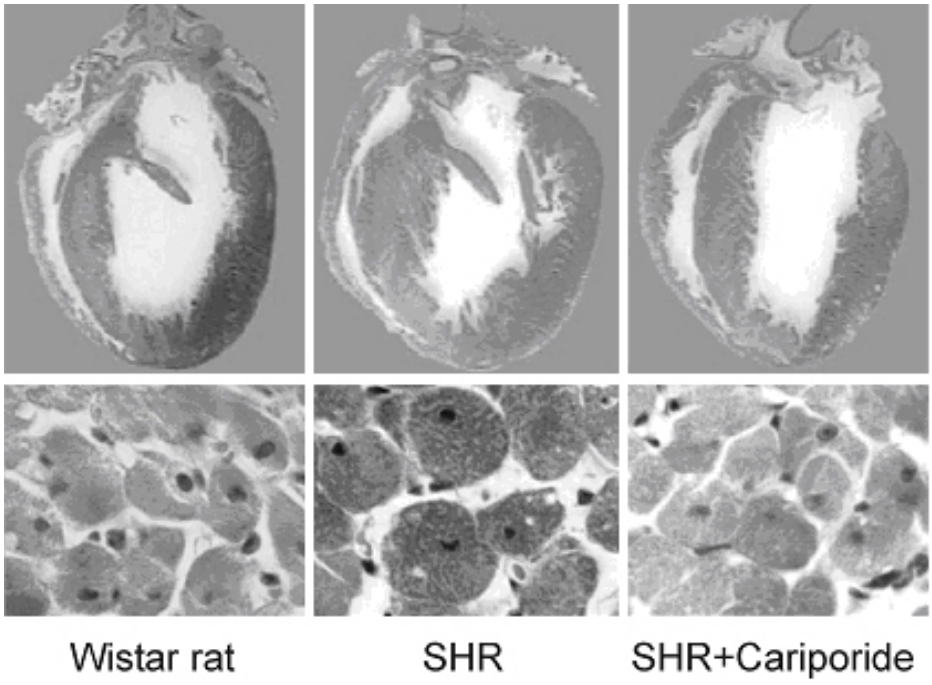

Figure 10. Chronic NHE-1 blockade with cariporide (one-month treatment) regressed myocardial hypertrophy in SHR. Upper panels show comparative major axis sections of representative hearts from a Wistar control rat (left), a non-treated SHR (middle) and a cariporide treated SHR (right), and lower panels show representative myocytes cross section micrographs from the three experimental groups. Modified from Camilion de Hurtado *et al.* (14) with permission.

models of cardiac hypertrophy. An up-regulation of NHE-1 was reported in cardiac hypertrophy and failure model of the β_1-adrenergic receptor transgenic mice (15). The inhibition of this exchanger prevented the development of cardiac hypertrophy and fibrosis, suggesting that NHE-1 was essential for the detrimental cardiac effects of chronic β_1-receptor stimulation in the heart (15). Similarly, cardiac hypertrophy induced in rats by chronic isoproterenol administration was prevented by inhibition of NHE-1 (16). On the other hand, we have also demonstrated that three different antihypertensive pharmacological interventions with different mechanisms of action (nifedipine, a Ca^{2+} channel blocker; enalapril, an inhibitor of angiotensin converting enzyme; and losartan, an AT_1 receptor blocker) caused the normalization of myocardial NHE activity, regression of cardiac hypertrophy, and decrease of arterial pressure in SHR (122). However, for a similar reduction in systolic blood pressure level and NHE activity, losartan induced the largest regression of cardiac hypertrophy. Even though these results give support to the hypothesis that an increased myocardial tension is determining intracellular signals having common end points on the antiporter activity and cellular growth, they also suggest that the eventual recruitment of additional intracellular pathways may be playing a role in the hypertrophic response.

The critical role of NHE-1 and/or $[Na^+]_i$ in the development of myocardial hypertrophy has been demonstrated in many different experimental models, like those mentioned below:

1) Hypertrophied hyperthyroid hearts show enhanced NHE-1 activity and when exposed to acute ischemia, they accumulate more Na^+ than the control non-hypertrophied hearts (17). These changes were prevented by NHE-1 inhibition (17). Furthermore, it has been demonstrated that thyroid hormone, by the interaction of its receptor with the NHE-1 promoter increases the expression of NHE-1 (123).

2) In patients with end-stage renal disease and secondary hyperparathyroidism as well as in patients with primary hyperparathyroidism, a strong correlation between cardiac hypertrophy and serum parathyroid hormone levels has been reported (124-126). This correlation was shown to be even much stronger than that between Ang II and hypertrophy (126). In addition, a direct evidence that parathyroid hormone improves hypertrophy was also reported (127). Though controversial (18,128), a stimulatory effect of parathyroid hormone on NHE-1 has been described; therefore, it is tempting to speculate about the possible involvement of the antiporter in the signaling pathway evoked by parathyroid hormone in the genesis of cardiac hypertrophy. On the other hand, low sodium plasma levels were detected in patients with NYHA class III-IV heart failure and high levels of parathyroid hormone (129). The resulting misbalance of the Na^+/Ca^{2+} may in turn be a factor to consider in the development of cardiac hypertrophy.

3) In rat neonatal ventricular myocytes, aldosterone stimulation induced a hypertrophic response accompanied by NHE-1 up-regulation and increased $[Na^+]_i$. Both

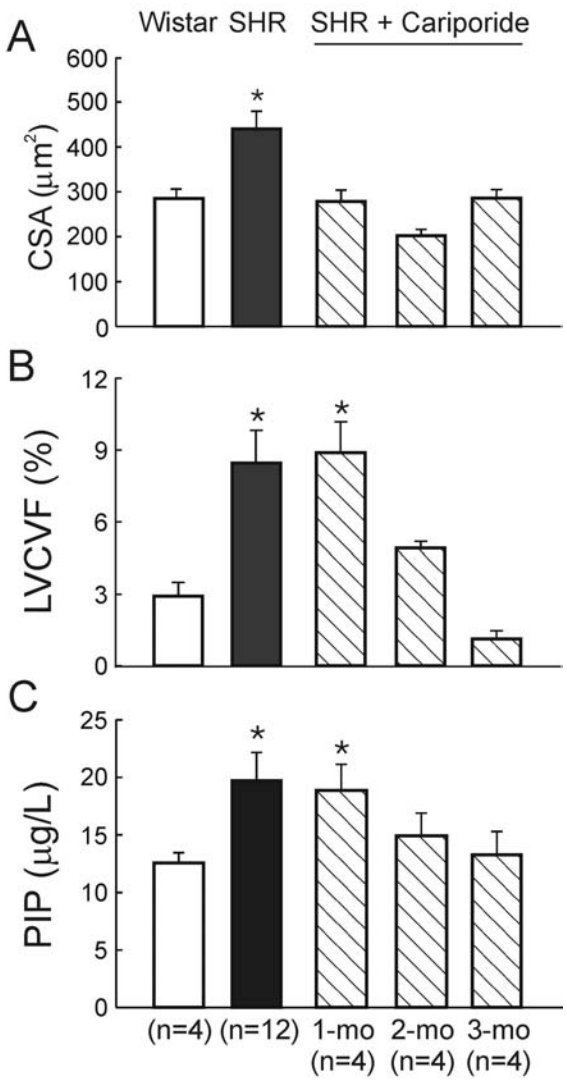

Figure 11. Chronic NHE-1 blockade normalized the enhanced interstitial fibrosis of the hypertrophic SHR hearts, but a longer treatment was necessary to observe this effect. Despite the fact that full regression of myocytes cross sectional area (CSA) was observed as early as after one-month cariporide treatment (Panel A), fibrosis indexes like left ventricle collagen volume fraction (LVCVF) (Panel B) and serum levels of the carboxyterminal propeptide of procollagen type I (PIP) (Panel C) remained elevated. However, when treatment duration was prolonged, normalization of fibrosis was observed (Panels B-C). Modified from Cingolani *et al.* (108) with permission.

hypertrophy and elevated (Na+)i were prevented by the NHE-1-specific inhibitor EMD87580 as well as the aldosterone antagonist spironolactone (19). Similar results were obtained in uninephrectomized rats exposed to deoxycorticosterone acetate/salt, in which cariporide treatment completely inhibited hypertrophy and NHE-1 up-regulation (20).

4) Recently a very interesting paper reported that cardiac hypertrophy of atrial natriuretic peptide receptor-deficient mice was accompanied by an increased activity of NHE-1, which thereby increased $[Ca^{2+}]_i$ (21). Those authors showed that these alterations were normalized by chronic treatment with the NHE-1 inhibitor cariporide. These results are in line with the report by Tajima *et al.* (130) demonstrating that atrial natriuretic peptide inhibits NHE-1 activity.

5) Emerging evidence indicates that leptin -a protein encoded by the obesity gene- is linked to cardiac hypertrophy (23-25). Interestingly, leptin has been reported to activate NHE-1 through a protein kinase C-dependent pathway (22). Moreover, it has been reported that leptin elevates ET-1 levels and, though speculative, this may be the pathway involved in NHE-1 stimulation (25). Furthermore, a recent report by Karmazyn's group implicated leptin as a mediator of hypertrophic effects of Ang II and ET-1 in cultured neonatal ventricular myocytes (24).

6) In right ventricular hypertrophy due to monocrotaline-induced pulmonary artery injury, myocardial NHE-1 expression was enhanced. As a consequence, both hypertrophy and NHE-1 up-regulation were abrogated by cariporide treatment (26).

7) When the sarcolemmal NHE-1 activity from normal unused human donor hearts was compared with that of recipient hearts with chronic end-stage heart failure exhibiting various degrees of hypertrophy, a significantly greater NHE-1 activity was detected in human hypertrophied myocytes (27).

As mentioned before, an enhanced activity of NHE-1 may be the result of an increased expression of the exchanger, an increased turnover of functional units, or a combination of both alternatives. In line with this, the reviewed models clearly exhibited cases of enhanced NHE-1 activity due to up-regulation, post-translational modification, or a combination of both. In either case, the hyperactivity of NHE-1 was linked to cardiac hypertrophy.

Interestingly, whereas chronic NHE-1 inhibition with cariporide in the whole animal induces up-regulation of the exchanger (131), the normalization of its previously augmented expression has been reported after chronic NHE-1 inhibition (15,16,21,26). Nevertheless, several aspects deserve further investigation to clarify the precise mechanism by which NHE-1 is involved in the development of cardiac hypertrophy and the possible link with other mechanisms of the intracellular hypertrophic program, like Wnt/Frizzled signaling pathways (132,133).

In line with the experiments reported by Kusumoto *et al.* (13) showing that NHE-1 inhibition decreased hypertrophy and remodeling after myocardial infarction, we have recently reported that post-myocardial infarction hypertrophy and fibrosis were reduced after phosphodiesterase 5A inhibition by sildenafil, the

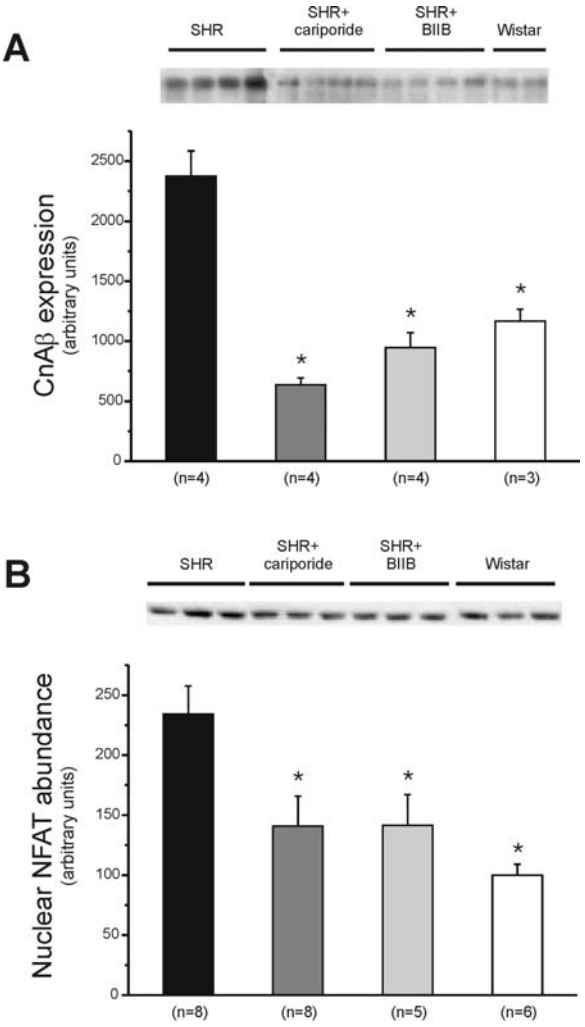

Figure 12. Hypertrophied myocardium of SHR showed an enhanced expression of CnAβ (Panel A) and an increased NFAT nuclear abundance (Panel B), compared to normotrophic myocardium of Wistar rats. Regression of cardiac hypertrophy promoted by two different NHE-1 inhibitors (cariporide and BIIB) was accompanied by normalization of both CnAβ expression (Panel A) and NFAT nuclear abundance (Panel B). Modified from Ennis *et al.* (118) with permission.

phosphodiesterase inhibition being accompanied by protein kinase G activation and NHE-1 inhibition (134).

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Abbreviations: Ang II= Angiotensin II, AT1= Angiotensin II receptor type 1, AT2= Angiotensin II receptor type 2, CnAb= b-isoform of calcineurin A, ERK1/2= extracellular signal-regulated protein kinases 1/2, ET= Endothelin, ET-1= Endothelin 1, ET-3= Endothelin 3, ETA= Endothelin type A receptors , mKATP= ATP-sensitive mitochondrial potassium , MPG= N- (2-mercaptopropionyl)-glycine, NADPH= Nicotinamide-Adenine Dinucleotide Phosphate, NCX= Na^+/Ca^{2+} exchanger, NCX_{rev}= Na^+/Ca^{2+} exchanger in reverse mode, NFAT= nuclear factor of activated T cells, NHE-1= cardiac Na^+/H^+ exchanger, p90rsk= p90 ribosomal S6 kinase, pH_i= intracellular pH, ROS= Reactive Oxygen Species, SHR= spontaneously hypertensive rats, $(Ca^{2+})_i$= intracellular Ca^{2+} concentration, $(Na^+)_i$= intracellular Na^+ concentration, $\cdot O_2^-$= superoxide anions

Key Words: NHE-1, Hypertrophy, Slow Force Response, Angiotensin II, Endothelin, Reactive Oxygen Species, Review

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