Myocardial Mineralocorticoid Receptor Activation by Stretching and Its Functional Consequences

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Abstract—Myocardial stretch triggers an angiotensin II–dependent autocrine/paracrine loop of intracellular signals, leading to reactive oxygen species–mediated activation of redox-sensitive kinases. Based on pharmacological strategies, we previously proposed that mineralocorticoid receptor (MR) is necessary for this stretch-triggered mechanism. Now, we aimed to test the role of MR after stretch by using a molecular approach to avoid secondary effects of pharmacological MR blockers. Small hairpin interference RNA capable of specifically knocking down the MR was incorporated into a lentiviral vector (l-shMR) and injected into the left ventricular wall of Wistar rats. The same vector but expressing a nonsilencing sequence (scramble) was used as control. Lentivirus propagation through the left ventricle was evidenced by confocal microscopy. Myocardial MR expression, stretch-triggered activation of redox-sensitive kinases (ERK1/2-p90^{RSK}), the consequent Na^+/H^+ exchanger–mediated changes in pH_i (HEPES-buffer), and its mechanical counterpart, the slow force response, were evaluated. Furthermore, reactive oxygen species production in response to a low concentration of angiotensin II (1.0 nmol/L) or an equipotent concentration of epidermal growth factor (0.1 μg/mL) was compared in myocardial tissue slices from both groups. Compared with scramble, animals transduced with l-shMR showed (1) reduced cardiac MR expression, (2) cancellation of angiotensin II–induced reactive oxygen species production but preservation of epidermal growth factor–induced reactive oxygen species production, (3) cancellation of stretch-triggered increase in ERK1/2-p90^{RSK} phosphorylation, (4) lack of stretch-induced Na^+/H^+ exchanger activation, and (5) abolishment of the slow force response. Our results provide strong evidence that MR activation occurs after myocardial stretch and is a key factor to promote redox-sensitive kinase activation and their downstream consequences. (***Hypertension*****. 2014;63:112-118.)** ● **Online Data Supplement**

Key Words: Na+/H+ exchanger ■ mineralocorticoid receptors ■ myocardial stretch

We recently reported that myocardial stretch induces the activation of the mineralocorticoid receptor (MR).[1] This conclusion was based on the prevention of some of the stretch-triggered consequences by 2 different MR inhibitors, spironolactone and eplerenone. These compounds canceled the stretch-induced activation of redox-sensitive kinases upstream of the Na^+/H^+ exchanger (NHE1), as well as the increase in NHE1 phosphorylation and its mechanical counterpart, the slow force response (SFR).[1–3] Previous studies demonstrated that inhibiting the NHE1 either pharmacologically or by silencing its expression also prevents these stretch-triggered actions[2–4] and decreases the production of mitochondrial reactive oxygen species (ROS).[5,6] However, pharmacological inhibition is not always deprived of nonspecific unwanted effects. Spironolactone is a nonselective compound that also blocks receptors other than the MR.[7] Furthermore, both spironolactone and eplerenone have been described as having an inverse agonist activity on the MR.[8] Therefore, we examined whether conclusive evidence about MR activation after myocardial stretch could be obtained using biomolecular techniques to silence MR expression in rat hearts. For this purpose, we took advantage of the RNA interference technique that we recently used to successfully blunt NHE1 expression in the rat myocardium.[4] Thus, small hairpin interfering RNA directed against the MR was incorporated into a lentiviral vector (l-shMR) and injected into the left ventricular wall of adult Wistar rats. One month later, the effect of stretching papillary muscles on the activation of redox-sensitive kinases, NHE1 activity, and SFR development, as well as the upstream signals mediating the increase in myocardial ROS production, was analyzed. Herein, we provide evidence supporting that myocardial stretch activates the MR, this step being critical for promoting the redox-sensitive kinase–mediated NHE1 stimulation. A preliminary report of our data has been presented elsewhere.[9]

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Materials and Methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine. Small hairpin interfering RNA capable of mediating specific MR knockdown was incorporated into a lentiviral vector (l-shMR) and injected into the left ventricular wall of Wistar rats. A lentiviral vector expressing a nonsilencing sequence (scramble) was used as control. A complete Materials and Methods section, including the detailed procedure to generate the interference RNA and inject this into the ventricular wall, is detailed in the online-only Data Supplement. That section also includes the methodology used to determine myocardial MR mRNA and protein expression, myocardial DsRed fluorescence by confocal microscopy, the stretch-triggered activation of the redox-sensitive kinases ERK1/2 and $p90^{RSK}$, the NHE1-mediated changes in pH_i, the SFR, and the ROS production.

Results

Lentivirus coding for the small hairpin interfering RNA-MR/DsRed (l-shMR) or the small hairpin interfering RNA-scramble sequence/dsRED (control) was injected into the rat myocardium at 2 sites in the left ventricular free wall near the cardiac apex, and rats were euthanized 1 month later. To check the spreading of the lentivirus, we measured DsRed fluorescence by confocal microscopy in the cardiac tissue from the site of injection to papillary muscles from l-shMR–injected, scramble-injected, and sham-operated (no injections) rats. A patchy red fluorescence distribution was observed in both groups of lentivirus-injected hearts, whereas no signal was detected in the sham-operated group (Figure 1). Magnified images highlighting the presence of DsRed inside the myocardial cells in a fascicular-like distribution pattern are shown in Figure S2 in the online-only Data Supplement.

MR protein expression measured in cardiac homogenates of the left ventricular myocardium, as well as in isolated papillary muscles, revealed that hearts injected with l-shMR had an ≈45% reduction in receptor expression compared with the control-injected hearts ($P<0.05$; Figure 2A and 2B). This result indicates that the small interfering RNA directed against the MR had spread throughout the ventricular myocardium from the sites of injection, as suggested by the confocal images shown in Figure 1. The injection of l-shMR did not modify the myocardial expression of either GAPDH (control: 100.1±9.8%, n=5; l-shMR: 100±5.3%, n=5) or another steroid receptor such as the glucocorticoid receptor (control: 100.0±7.7%, n=4; l-shMR: 90.9±8.9%, n=4), suggesting that the l-shMR vector specifically targeted the MR, minimizing the possibility of nonspecific effects. Consistent with the reduction in MR protein expression, hearts injected with l-shMR had an ≈55% reduction in MR mRNA abundance compared with those injected with the control vector (Figure 2C). Importantly, no changes in MR protein expression were detected either in the lung or in the liver of injected animals (Figure S3), supporting the idea that the presence of l-shMR was restricted to the myocardium. Similar results were obtained when this technique was used to silence the NHE1 by direct injection of a naked interference RNA in the left ventricular wall.[10]

Because we have previously shown that the SFR to myocardial stretch is the mechanical counterpart of an autocrine/paracrine mechanism triggered by the release of endogenous

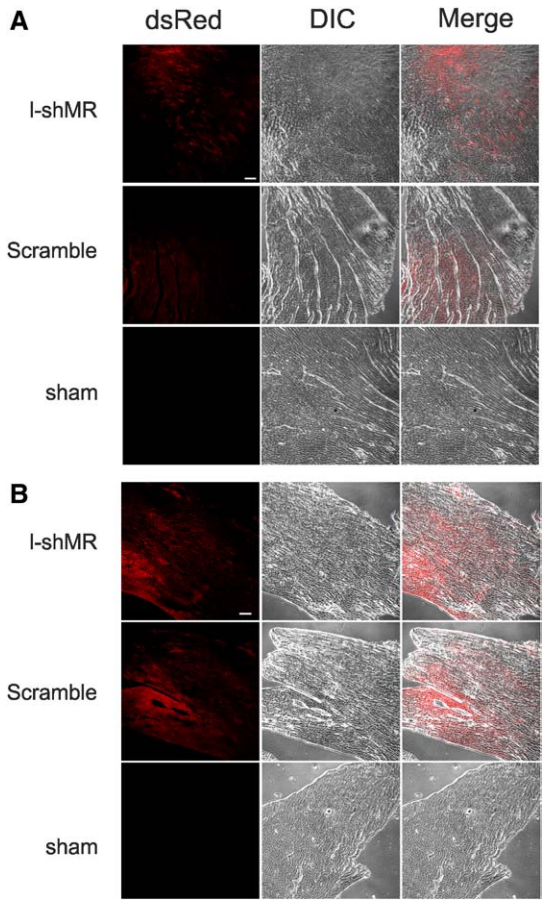

Figure 1. Myocardial patchy distribution of the lentivirus. **A**, Representative confocal images of lentiviral vector (l-shMR)–injected (**top**), scramble-injected (**middle**), and sham-operated (**bottom**) rat myocardium. Samples of the myocardium were serially sectioned using a vibratome. The first column shows the DsRed fluorescence. The second column presents the captured myocardial area using differential interference contrast (DIC). The third column represents the merging of the previous images. Different intensities of DsRed expression and a patchy distribution could be appreciated in the first and second rows. **B**, Same as **A** but in papillary muscles. Scale bar, 100 μm.

angiotensin II (Ang II) involving MR and NHE1 activation,[11] we decided to functionally test the MR under our experimental conditions by exploring the SFR and NHE1 activation after stretching isolated papillary muscles from l-shMR– and control-injected hearts. The stretching of the papillary muscles in the control group promoted the characteristic biphasic mechanical response, an initial abrupt force increase followed by the SFR (Figure 3A–3C). On the contrary, the SFR was absent in the papillary muscles isolated from MR-silenced hearts (Figure 3B and 3C).

Alkalization of cardiac muscle in bicarbonate-free medium is a key signal of stretch-triggered NHE1 activation[12]; therefore, we next determined pH_i changes after stretching isolated papillary muscles from both experimental groups. As expected, a significant increase in pH_i was detected in papillary muscles from control-injected hearts, whereas no pH_i changes were observed in the l-shMR–injected group as shown in Figure 4, revealing the absence of stretch-induced NHE1 activation. The results shown in Figures 3 and 4 indicate that MR activation

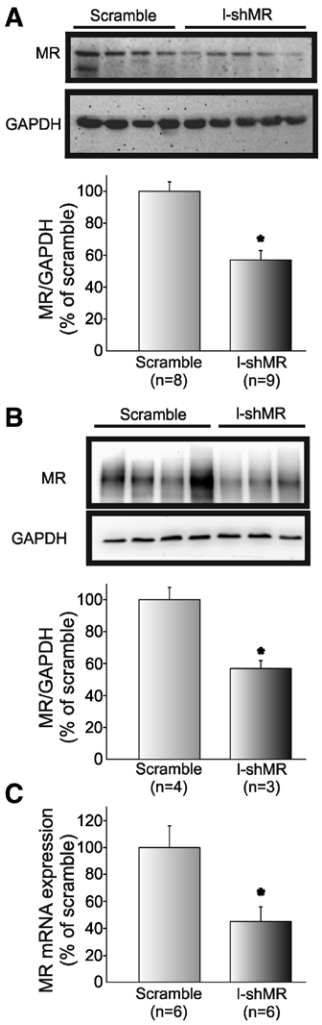

Figure 2. Mineralocorticoid receptor (MR) protein and mRNA expression in ventricular myocardium and papillary muscles. Hearts injected with lentiviral vector (l-shMR) designed to specifically downregulate MR expression showed a significant reduction in the amount of receptor protein compared with the scramble-injected hearts as shown in the representative original Western blot and in the averaged results of **A** (left ventricular homogenates) and **B** (papillary muscles). Consistently, hearts injected with l-shMR had an ≈55% reduction in MR mRNA abundance compared with scramble-injected heart. **P*<0.05 vs scramble (**C**).

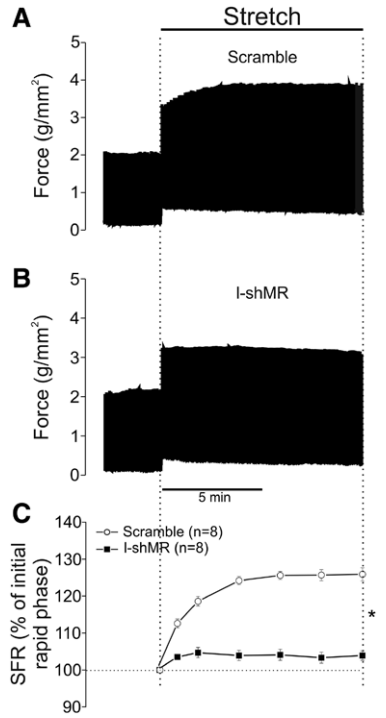

Figure 3. Slow force response (SFR) and mineralocorticoid receptor (MR) activation. **A**, Original force record of a papillary muscle from a scramble-injected rat heart subjected to an increase in length from 92% to 98% of L_{max}, where it can be appreciated the classical biphasic response to stretch. **B**, Same as **A** but of a papillary muscle from a lentiviral vector (l-shMR)–injected rat heart where the SFR was suppressed. **C**, Averaged SFR expressed as percentage of the initial rapid phase from both groups of animals. **P*<0.05 scramble vs l-shMR.

is crucial to NHE1 stimulation and the SFR development after myocardial stretching, as reported previously based on pharmacological interventions.[1]

Myocardial stretch induces NHE1 phosphorylation at Ser703[13] through a mechanism that involves redox-induced activation of ERK1/2 and p90^{RSK}.[13,14] As expected, we detected a significant increase in ERK1/2 and p90^{RSK} phosphorylation after stretching control-injected papillary muscles, an effect that was considerably blunted in muscles from MR-silenced hearts (Figure 5). Therefore, an activated MR is a necessary condition to induce redox-sensitive kinase activation after myocardial stretch.

As we have already mentioned, myocardial stretching induces the activation of the Ang II AT1 receptor, triggering a chain of autocrine/paracrine intracellular signals that through the activation of the epidermal growth factor receptor (EGFR) leads to increased mitochondrial ROS production and activation of redox-sensitive kinases (Figure 5).[13] To further characterize the signals upstream of these kinases, measurement of myocardial $O_2^{\cdot}$ production after a low concentration of Ang II (1 nmol/L), which was previously shown to mimic the increase in force observed during the SFR,[15] was compared in tissue slices obtained from control- and l-shMR–injected hearts. Figure 6 shows that Ang II increased $O_2^{\cdot}$ production by ≈50% in the control group, whereas this effect was not observed in the myocardial slices from MR-silenced hearts. However, when an equipotent concentration of EGF (0.1 μg/mL) was used to stimulate $O_2^{\cdot}$

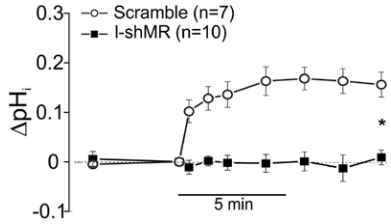

Figure 4. Intracellular pH changes after stretch in isolated papillary muscles bathed with bicarbonate-free solution. As expected, myocardial stretch increased pH_i in papillary muscles from scramble-injected hearts demonstrating the stretch-triggered Na^+/H^+ exchanger activation, an effect that was not detected in muscles from lentiviral vector (l-shMR)–injected hearts. Averaged results expressed as ΔpH_i. **P*<0.05 scramble vs l-shMR.

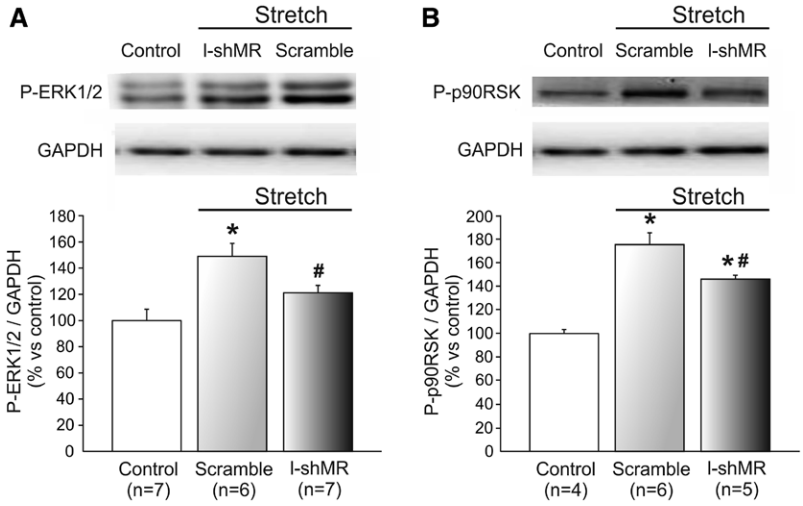

Figure 5. ERK1/2 and p90[RSK] phosphorylation after stretch. The stretch of scramble-injected papillary muscles significantly increased ERK1/2 (**A**) and p90[RSK] (**B**) phosphorylation expressed as percentage of nonstretched muscles, effect that was significantly blunted in papillary muscles from lentiviral vector (l-shMR)–injected hearts. Representative Western blots (**top**) and averaged P-ERK1/2 or P-p90[RSK] to GAPDH ratios (**bottom**). **P*<0.05 vs control, #*P*<0.05 vs scramble.

formation, MR silencing did not abrogate the increase in $O_2^{\cdot}$ production observed in the control group (Figure 6), suggesting that the MR is located downstream of the AT1 but upstream of the EGFR in the mitochondrial ROS formation pathway. Taken together, these data unequivocally support that MR activation after myocardial stretch is an important event upstream of myocardial ROS production and ERK1/2, p90[RSK], and NHE1 activation.

Discussion

In the present study, a gene-silencing strategy with small interfering RNA was used to specifically downregulate MR protein expression in the rat myocardium. This approach reduced MR mRNA and protein expression to approximately half in homogenates of the whole left ventricle, as well as in the papillary muscles used for functional studies, an effect that was enough to blunt the stretch-triggered activation of redox-sensitive kinases upstream of the NHE1 and its downstream effects. These results come to extend the relevance of our previous report using eplerenone to prevent MR activation after stretch[1] by providing strong support for the notion that MR activation is a consequence of the intracellular signals triggered by myocardial stretch. The effectiveness of the technique used to silence the myocardial MR seems to be related to the spread of the shMR from cell to cell through connexins, as proposed by Kizana et al.[16] Furthermore, we successfully used this same methodology in a previous work to knockdown the NHE1 expression in rat hearts.[4] In addition, Gupta et al[17] used this technique to silence nuclear factor-κβ, and Schuman et al[18] used this technique to identify the key role of cardiac thyrotropin-releasing hormone in mediating left ventricular hypertrophy in spontaneously hypertensive rats.

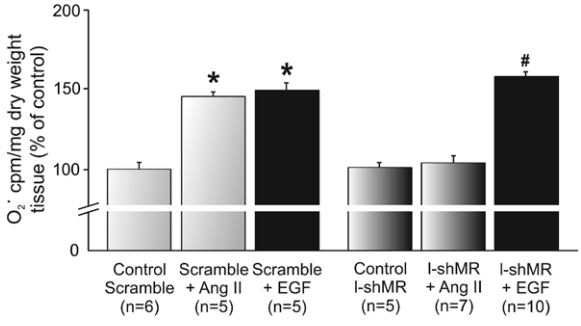

Figure 6. Myocardial superoxide anion production induced by angiotensin II (Ang II) or epidermal growth factor. Ang II increased $O_2^{\cdot}$ production by ≈50% of control in the myocardium of scramble-injected hearts, an effect that was canceled in the mineralocorticoid receptor (MR)–silenced group. This result indicates that Ang II requires the activation of the MR to promote an increase in $O_2^{\cdot}$ production in the myocardium. In contrast, when epidermal growth factor (EGF) was used to stimulate $O_2^{\cdot}$ formation, the increase in $O_2^{\cdot}$ production was observed in both experimental groups, indicating that in the route of reactive oxygen species formation the MR is located upstream of the EGF receptor. Results are expressed as percentage of the corresponding control (scramble or lentiviral vector [l-shMR]) without interventions. **P*<0.05 vs control scramble, #*P*<0.05 vs control l-shMR.

The reduction in MR expression at both the protein and mRNA levels found in homogenates of the whole left ventricle of l-shMR–injected hearts was only partial but sufficient to completely prevent the stretch-induced functional effects, proving the effectiveness of the MR-silencing procedure. The modest decrease in MR protein expression that we detected could possibly be a result of insufficient delivery of the lentiviral-transducing units. However, as quoted before, a complete functional inactivation of the receptor was observed. Whether this effect represents a characteristic of MR function, in which 50% of a decrease in the functional units is enough to achieve complete cancellation of the increase in ROS production and its downstream effects, is not apparent to us at this moment.

A link between Ang II or its AT1 receptor and the MR has been reported previously.[19–21] In connection with this, the release of preformed Ang II by the cardiomyocytes after stretch was proved in elegant experiments by Sadoshima et al[22] and later on by Leri et al.[23] In addition, Browe and Baumgarten[24] proposed a mechanotransduction model explaining the release of Ang II after stretch, where β1 integrin activation may conceivably be the link between membrane deformation and the release of endogenous Ang II. However, we have previously shown that AT1 receptor blockade with losartan blunts the SFR to stretch,[2,3] and this mechanical effect can

be mimicked by a low concentration of exogenous Ang II[15] (within the concentration range reported to be released after myocyte stretching by Sadoshima and Izumo).[22] In the present study, we show that the Ang II–promoted increase in ROS production is also canceled in the MR-silenced myocardium, which is in line with previous reports in smooth and cardiac muscles.[1,21] However, increased ROS formation in response to EGF was preserved in the myocardium where the MR expression was downregulated. This suggests that in the chain of intracellular signals after myocardial stretch, the role of the MR is upstream of the EGFR and that MR activation is necessary to stimulate the downstream functional effects, confirming our previous findings using either pharmacological inhibition of the MR or a metalloproteinase inhibitor to prevent MR-dependent EGFR transactivation.[1] Furthermore, these results reinforce the notion that EGFR transactivation occurs via the MR[1] and not directly through a G protein–coupled receptor as we proposed previously.[13] The fact that EGFR transactivation can be triggered by MR activation was recognized previously by others.[19,25,26] EGFR transactivation seems to be performed by metalloproteinase-dependent cleavage of proheparin-binding EGF, which promotes ectoshedding of heparin-binding EGF.[27,28] Heparin-binding EGF is a well-known ligand of EGFR, and binding of heparin-binding EGF to EGFR leads to activation of the receptor by tyrosine autophosphorylation. Once EGFR is phosphorylated, the downstream signals increase mitochondrial $O_2^{\cdot}$ production. Figure 7 illustrates our hypothesis in which transactivation takes place after metalloproteinase activation subsequent to MR stimulation.

In the present study, we did not explore the mechanism by which myocardial MR becomes activated after stretching. Aldosterone synthesis by the cardiac myocyte is unlikely in light of previous reports showing that the heart has no or extremely low mRNA of aldosterone synthase and 11-β-hydroxilase.[29–31] However, myocardial aldosterone was detected in 30% of hearts from adrenalectomized rats,[29] suggesting that the heart is probably able to synthesize small amounts of aldosterone. In any case, aldosterone uptaken by the heart from the circulation, synthesized by the heart, or a combination of both could be released after stretch. It is important to keep in mind that Ang II is the primary stimulus for aldosterone production from the adrenal glands, and pharmacological amounts of exogenous Ang II were reported to increase the aldosterone concentration in the heart and serum.[32]

The MR is overexpressed in heart failure,[33,34] and the clinical efficacy of MR inhibitors in the treatment of heart failure has been well established.[35–37] However, the exact mechanisms by which MR antagonists provide cardiovascular protection in patients with cardiac failure are not completely understood. From a simplistic point of view, it may be reasoned that elevated circulating levels of aldosterone, resulting in activation of MR in different tissues, could be detrimental; therefore, MR blockade would be beneficial. However, this argument is challenged by the fact that the beneficial effects of MR antagonism are observed even at normal circulating levels of aldosterone[35,38–40] and are independent of natriuretic effects.[35,39,40] Furthermore, under normal conditions without an increase in oxidative stress, the MR is occupied but not activated by glucocorticoids.

As we reported previously[1] and confirm here in the control-injected hearts (Figure 6), MR activation increases ROS formation. Therefore, an expected consequence of

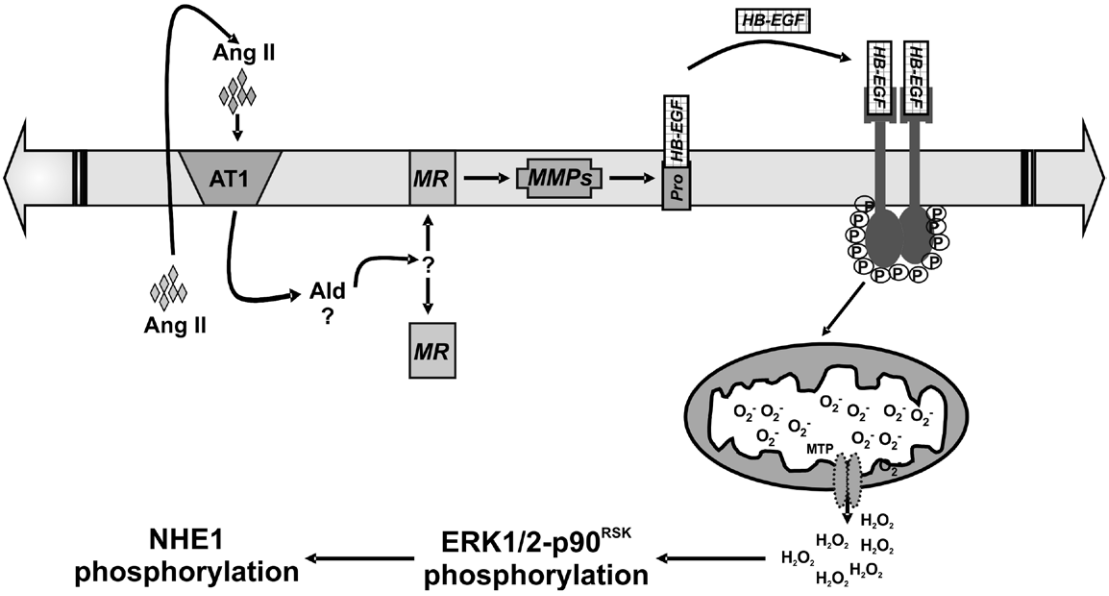

Figure 7. Suggested sequence of events triggered by myocardial stretch. Mineralocorticoid receptor (MR) activation, probably by endogenous formation/release of aldosterone, seems to be located upstream of epidermal growth factor receptor (EGFR) activation in the chain of intracellular signals, leading to the slow force response. Therefore, EGFR transactivation seems to originate from MR and not from G protein–coupled receptor, as we suggested previously. EGFR transactivation seems to result from a metalloproteinase (MMPs)-promoted ectoshedding of heparin-binding EGF (HB-EGF), a known ligand of EGFR, which promotes receptor activation by tyrosine autophosphorylation, leading to mitochondrial reactive oxygen species formation, redox-sensitive kinase activation, and Na^+/H^+ exchanger (NHE1) phosphorylation. Ang II indicates angiotensin II.

the increased oxidative stress should be the enhanced function of the NHE1, as we recently reported.[41] Interestingly, a role for the NHE1 in heart failure was proposed by Baartscheer et al,[42] who in elegant experiments showed that long-term NHE1 inhibition with cariporide decreased the augmented diastolic calcium without significant alteration in systolic calcium in rabbits with hypertrophy and failure. The decrease in diastolic calcium is probably sensed by the prohypertrophic phosphatase, calcineurin, whereas the increase in the calcium transient amplitude increases cardiac inotropism.

Our data suggest that MR antagonism may be followed by inhibition of mitochondrial ROS formation and NHE1 activation, 2 known deleterious signals for the heart. Interestingly, these actions could be responsible, at least in part, for the salutary effects of the MR inhibitors used clinically in heart failure,[35–37] where an increase in oxidative stress takes place. Our results are in line with previous reports in mice in which ablation of the MR in myocytes preserves cardiac function after chronic pressure overload[43] and also decreases adverse cardiac remodeling after myocardial infarction,[44] 2 pathological conditions in which the myocardium is stretched. A similar protection was obtained when mice deprived of myocardial MR were compared with their controls after treatment with deoxycorticosterone acetate/salt.[45]

Perspectives

There is growing evidence showing striking beneficial effects of MR blockade in the treatment of systolic heart failure, a condition in which cardiac muscle is stretched. Despite that these salutary effects have been well documented in 3 different clinical trials (RALES,[35] EPHESUS,[36] and EMPHASIS[37]), the exact mechanism by which MR inactivation improves heart failure is still not clear. In the present study, by silencing cardiac MR expression, we confirmed its activation after myocardial stretch. MR activation is a step in a chain of events leading to EGFR transactivation, enhanced mitochondrial ROS production, redox-sensitive kinase activation, and NHE1 activation. We can speculate that the decrease in ROS formation after the deactivation of the MR-triggered signaling cascade may be responsible for at least part of the beneficial effects of the clinically used MR blockers in cardiac failure.[35–37]

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Disclosures

None.

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Novelty and Significance

What Is New?

- We report mineralocorticoid receptor (MR) activation after myocardial stretch.

What Is Relevant?

- Because stretched myocardium is a characteristic of systolic heart failure in which an increase in oxidative stress occurs, silencing of the MR would be beneficial. Our data suggest that MR inhibition is followed by deactivation of mitochondrial reactive oxygen species formation and Na^+/H^+ exchanger stimulation, 2 known deleterious signals of the heart. These actions could be responsible, at least in part, for the salutary effects of the MR inhibitors in patients with heart failure.

Summary

In this study, a gene-silencing strategy was used to specifically downregulate MR protein expression in the myocardium to specifically test the role of MR activation after stretch. Our findings provide strong evidence that MR activation occurs after myocardial stretch and is a key factor promoting redox-sensitive kinase activation and their downstream consequences.