Aldosterone Stimulates the Cardiac Na^+/H^+ Exchanger via Transactivation of the Epidermal Growth Factor Receptor

Verónica C. De Giusti, Mariela B. Nolly, Alejandra M. Yeves, Claudia I. Caldiz, María C. Villa-Abrille, Gladys E. Chiappe de Cingolani, Irene L. Ennis, Horacio E. Cingolani, Ernesto A. Aiello

Abstract—The use of antagonists of the mineralocorticoid receptor in the treatment of myocardial hypertrophy and heart failure has gained increasing importance in the last years. The cardiac Na^+/H^+ exchanger (NHE-1) upregulation induced by aldosterone could account for the genesis of these pathologies. We tested whether aldosterone-induced NHE-1 stimulation involves the transactivation of the epidermal growth factor receptor (EGFR). Rat ventricular myocytes were used to measure intracellular pH with epifluorescence. Aldosterone enhanced the NHE-1 activity. This effect was canceled by spironolactone or eplerenone (mineralocorticoid receptor antagonists), but not by mifepristone (glucocorticoid receptor antagonist) or cycloheximide (protein synthesis inhibitor), indicating that the mechanism is mediated by the mineralocorticoid receptor triggering nongenomic pathways. Aldosterone-induced NHE-1 stimulation was abolished by the EGFR kinase inhibitor AG1478, suggesting that is mediated by transactivation of EGFR. The increase in the phosphorylation level of the kinase $p90^{RSK}$ and NHE-1 serine703 induced by aldosterone was also blocked by AG1478. Exogenous epidermal growth factor mimicked the effects of aldosterone on NHE-1 activity. Epidermal growth factor was also able to increase reactive oxygen species production, and the epidermal growth factor–induced activation of the NHE-1 was abrogated by the reactive oxygen species scavenger *N*-2-mercaptopropionyl glycine, indicating that reactive oxygen species are participating as signaling molecules in this mechanism. Aldosterone enhances the NHE-1 activity via transactivation of the EGFR, formation of reactive oxygen species, and phosphorylation of the exchanger. These results call attention to the consideration of the EGFR as a new potential therapeutic target of the cardiovascular pathologies involving the participation of aldosterone. **(*Hypertension*. 2011;58:912-919.) ● Online Data Supplement**

Key Words: cardiac myocytes ■ sodium/hydrogen exchanger ■ aldosterone ■ epidermal growth factor receptor ■ transactivation

The investigation of the role of aldosterone in cardiovascular pathophysiology has gained increasing interest in the last few years because of relevant results obtained from clinical studies, particularly the Randomized Aldactone Evaluation Study, Eplerenone Postacute Myocardial Infarction Heart Failure Efficacy and Survival Study, and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure, in which antagonists of the mineralocorticoid receptor (MR) importantly reduced mortality in patients with left ventricular dysfunction.[1–4] Aldosterone has been shown to induce left ventricular hypertrophy independently from its classic effects on regulation of renal Na^+ and blood pressure.[5–7] However, the cellular, subcellular, and molecular bases for this effect are not yet understood. Fujisawa et al[8] demonstrated that the mineralocorticoid/salt-induced rat cardiac fibrosis and hypertrophy were prevented by the selective Na^+/H^+ exchanger (NHE-1) blocker cariporide. It has also been reported that aldosterone upregulates the expression and function of the NHE-1[9–12] and that selective blockade of this transporter prevents and/or reverts left ventricular hypertrophy in various animal models.[13] Therefore, we focused our attention on the intracellular pathway that involves aldosterone and NHE-1 as trigger and end point target, respectively.

Steroids, including aldosterone, are able to interact with peptide hormone signaling. The epidermal growth factor (EGF) and its receptor (EGFR) represent one of these signals involving aldosterone.[14,15] It has been shown that spironolactone reduces the EGFR mRNA synthesis after cerebral ischemia.[16] Accordingly, Grossmann et al[15] reported that MR activation by aldosterone enhanced EGFR expression via an interaction with the EGFR promoter of vascular smooth muscle. In addition to the genomic effects, nongenotropic actions of aldosterone concerning EGFR transactivation have also been reported.[17,18]

The EGFR belongs to the ErbB family of tyrosine kinase membrane receptors that is composed of 4 members, EGFR/

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From the Centro de Investigaciones Cardiovasculares (CONICET), Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina.

Correspondence to Ernesto A. Aiello, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, UNLP-CONICET, Calle 60 y 120, 1900 La Plata, Argentina. E-mail aaiello@med.unlp.edu.ar

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ErbB1/HER1, ErbB2, HER2/neu, ErbB3/HER3, and ErbB4/HER4.[19] On ligand binding, the autophosphorylation of specific tyrosine residues in the cytosolic domain of the receptor takes place triggering a downstream signaling pathway. Ligands for the EGFR include EGF, transforming growth factor-α, heparin-binding EGF (HB-EGF), and amphiregulin. HB-EGF is expressed as a transmembrane precursor and released from the cell surface after shedding of the extracellular domain (ectodomain shedding) by zinc-dependent metalloproteinases.[19] This ligand is the mediator of EGFR transactivation by G protein–coupled receptors. Aldosterone is a known stimulus for matrix metalloproteinases.[17] Among the effects proposed to result from aldosterone-induced EGFR transactivation, Mazak et al[20] determined in vascular smooth muscle cells that aldosterone induces the phosphorylation of extracellular signal–regulated kinase (ERK) 1/2 kinase through EGFR activation. Interestingly, myocardial hypertrophy has been linked to this intracellular signaling pathway.[21] Because it has been demonstrated that EGF also stimulates NHE-1 in different cell types,[22–24] including cardiac myocytes,[25] we hypothesized that aldosterone would be able to activate this transporter via transactivation of the EGFR. Herein, we present evidence that confirm this hypothesis.

Methods

All of the procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85-23, revised 1996), and the experimental protocol was approved by the animal welfare committee of La Plata School of Medicine. Rats (body weight: 300–400 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg of body weight) and hearts rapidly excised when plane 3 of phase III of anesthesia was reached.

Cell Isolation

Rat ventricular myocytes were isolated according to the technique described previously[26] from 4-month–old Wistar rats.

Intracellular pH Measurements

Intracellular pH (pH_i) was measured in single myocytes with an epi-fluorescence system (Ion Optix, Milton, MA) using the previously described 2′-7′-bis(carboxyethyl)-5(6)-carboxyfluorescein technique.[27]

Determination of p90^{RSK} and NHE-1 Phosphorylation

p90^{RSK} and NHE-1 phosphorylation were determined by Western blots with phosphospecific antibodies as described previously.[28]

Measurements of Superoxide Anion Production

We used lucigenin-enhanced chemiluminescence to measure superoxide anion production by rat cardiac tissue in Krebs-Hepes buffer with 5 μmol/L of lucigenin, as described previously.[29] An expanded Methods section is available in the online Data Supplement at http://hypertension.ahajournals.org.

Results

Effect of Aldosterone on NHE-1 Activity: Role of the MR

We investigated the effect of aldosterone on acute NHE-1 activity during the recovery of an acid load in rat ventricular myocytes. Figure 1A shows the effect of exposing a rat

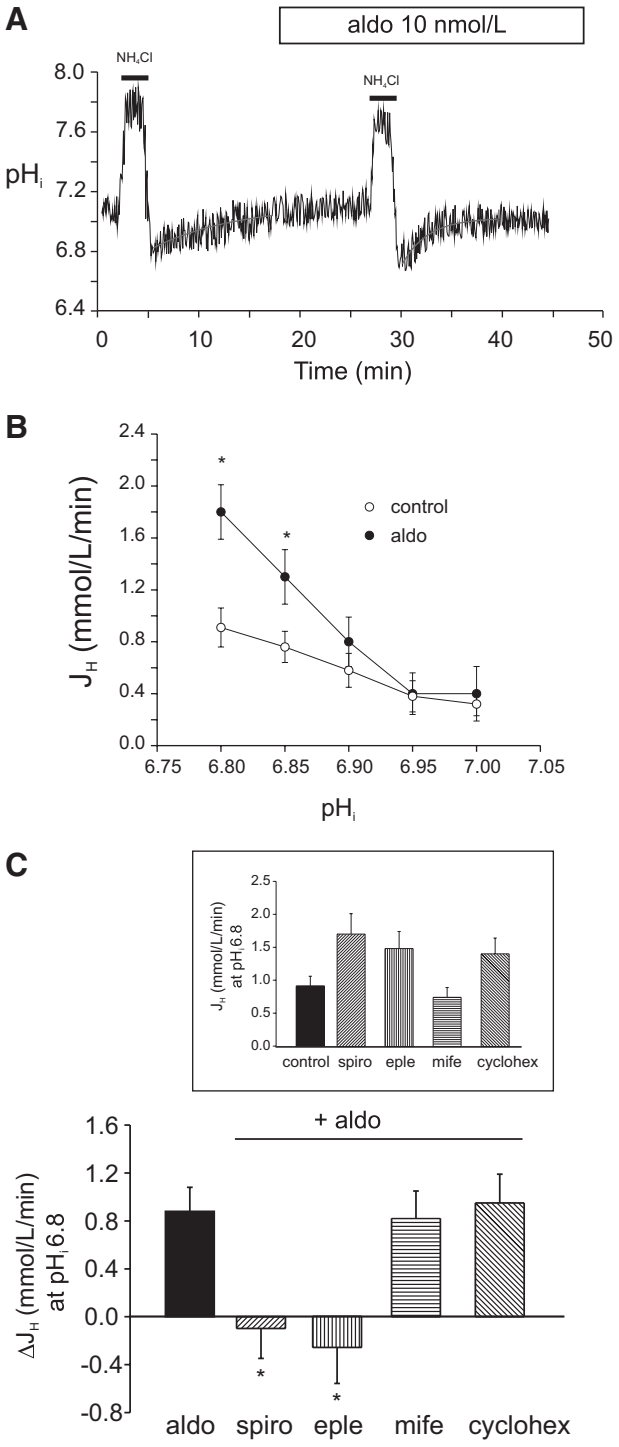

Figure 1. Aldosterone induced activation of the Na^+/H^+ exchanger (NHE-1). **A**, Representative traces of intracellular pH (pH_i) during the application of 2 consecutive ammonium pulses (20 mmol/L of NH_4Cl), in the absence (first pulse) and presence of 10 nmol/L of aldosterone (aldo; second pulse). Aldo was applied 10 minutes before the second pulse. **B**, Average proton efflux (J_H), carried by the NHE-1, before (first pulses; ○; n=5) and after application of 10 nmol/L of aldo (second pulses; ●; n=5). *$P<0.05$ vs control. **C**, Average increase in J_H at pH_i 6.8 expressed as the difference between the second and the first pulse, in the presence of 10 nmol/L of aldo (n=5) and in the presence of aldo plus spironolactone (spiro; 10 μmol/L; n=5), plus eplerenone (eple; 10 μmol/L; n=6), plus mifepristone

ventricular myocyte to aldosterone (10 nmol/L) 10 minutes before the beginning of the ammonium pulse. The hormone significantly increases the proton efflux (J_H) at acidic pH_i (Figure 1B). According to a rapid effect, preincubation of the myocytes with cycloheximide (inhibitor of protein synthesis, 10 μmol/L) did not prevent the stimulatory effect of aldosterone on NHE-1–mediated pH_i recovery (Figure 1C). The effect of aldosterone was abolished by pretreatment of the cells with the MR antagonists spironolactone (10 μmol/L) or eplerenone (10 μmol/L), indicating that the MR participates in such rapid effect of aldosterone (Figure 1C). In addition, mifepristone (glucocorticoid receptor antagonist, 1 μmol/L) did not affect the aldosterone-mediated NHE-1 stimulation, confirming that aldosterone specifically activated the MR (Figure 1C). Although higher basal J_H was observed with spironolactone, eplerenone, and cycloheximide (Figure 1C, inset), no statistical significance was attained with any of the drugs used. To confirm that spironolactone (which produced the greatest effect on basal J_H) was not truly significantly affecting basal J_H, we performed experiments in which we added spironolactone only to the second ammonium pulse, the first one being the control in the absence of the drug. Under these conditions, no effect of spironolactone was detected (ΔJ_H at pH_i 6.8: 0.09±0.25 mmol/L per minute; n=5), further indicating that this drug does not affect basal J_H.

Involvement of EGFR Transactivation in Aldosterone-Induced NHE-1 Stimulation

To investigate whether the aldosterone-induced NHE-1 stimulation is mediated by EGFR transactivation, the myocytes were pretreated with AG1478 (EGFR kinase inhibitor, 1 μmol/L) 10 minutes before the addition of aldosterone. Figure 2A and 2B shows that AG1478 prevented aldosterone-induced NHE stimulation during pH_i recovery from acidosis. As mentioned above, EGF is released from the cell surface by zinc-dependent metalloproteinases[19] and mediates the EGFR transactivation by G protein–coupled receptors. Aldosterone is a known stimulus for matrix metalloproteinases.[17] Thus, we measured pH_i in the presence of the inhibitor of the metalloproteinases, matrix metalloproteinase inhibitor II. This compound inhibited the effects of aldosterone (Figure 2C), indicating that this hormone is promoting the release of EGF from the cell, which, in turn, activates the EGFR. Because the src-kinase is known to be involved in EGFR transactivation in various scenarios,[14] we evaluated the effect of aldosterone in the presence of PP1, a selective inhibitor of src-kinase. Under these conditions, aldosterone failed to stimulate the NHE-1 (Figure 2C), suggesting that the src-kinase participates in the EGFR transactivation promoted by the steroid.

Figure 1 (Continued). (miphe; 1 μmol/L; n=4), or plus cycloheximide (ciclohex; 10 μmol/L; n=5). *$P<0.05$ vs aldo. Inset, Average absolute values of basal J_H (recovery from the first of the 2 consecutive ammonium pulses) at pH_i 6.8 recorded in the absence (control) or presence of spironolactone, eplerenone, mifepristone, or cycloheximide.

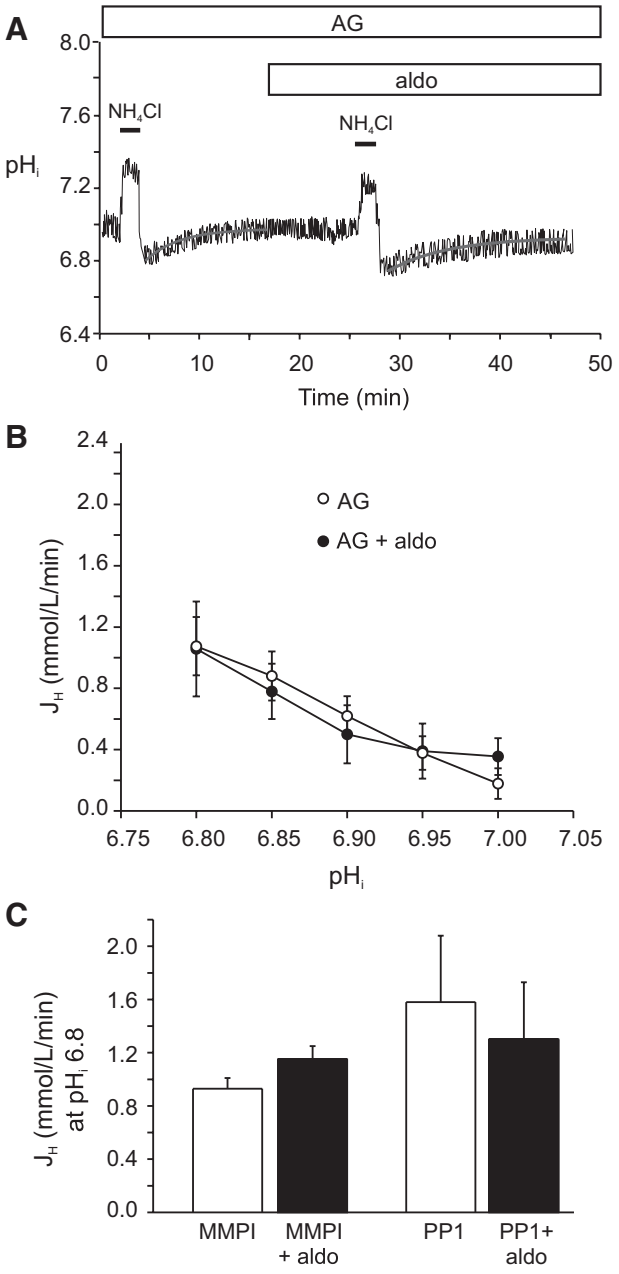

Figure 2. A, Representative traces of intracellular pH (pH_i) during the application of 2 consecutive ammonium pulses (20 mmol/L of NH_4Cl), in the absence (first pulse) and presence of 10 nmol/L of aldosterone (aldo; second pulse). AG1478 (AG; 1 μmol/L) was applied 10 minutes before the first pulse and maintained throughout the experiment. Aldo was applied 10 minutes before the second pulse. **B**, Average proton efflux (J_H), carried by the Na^+/H^+ exchanger (NHE-1), before (first pulses; ○; n=4) and after application of 10 nmol/L of aldo (second pulses; ●; n=4) in the continuous presence of 1 μmol/L of AG. **C**, Average J_H obtained at pH_i of 6.8 in the presence of the matrix metalloproteinase inhibitor II (3 μmol/L; n=5) or the src-kinase blocker PP1 (20 μmol/L; n=5) in the absence and presence of aldo (10 nmol/L).

Effects of Exogenous EGF on NHE-1 Activity

To confirm that EGFR activation was able to stimulate NHE-1 activity in cardiac myocytes, we pretreated the cells with exogenous EGF (0.1 μg/mL). Figure 3 shows that the addition of EGF to the extracellular medium

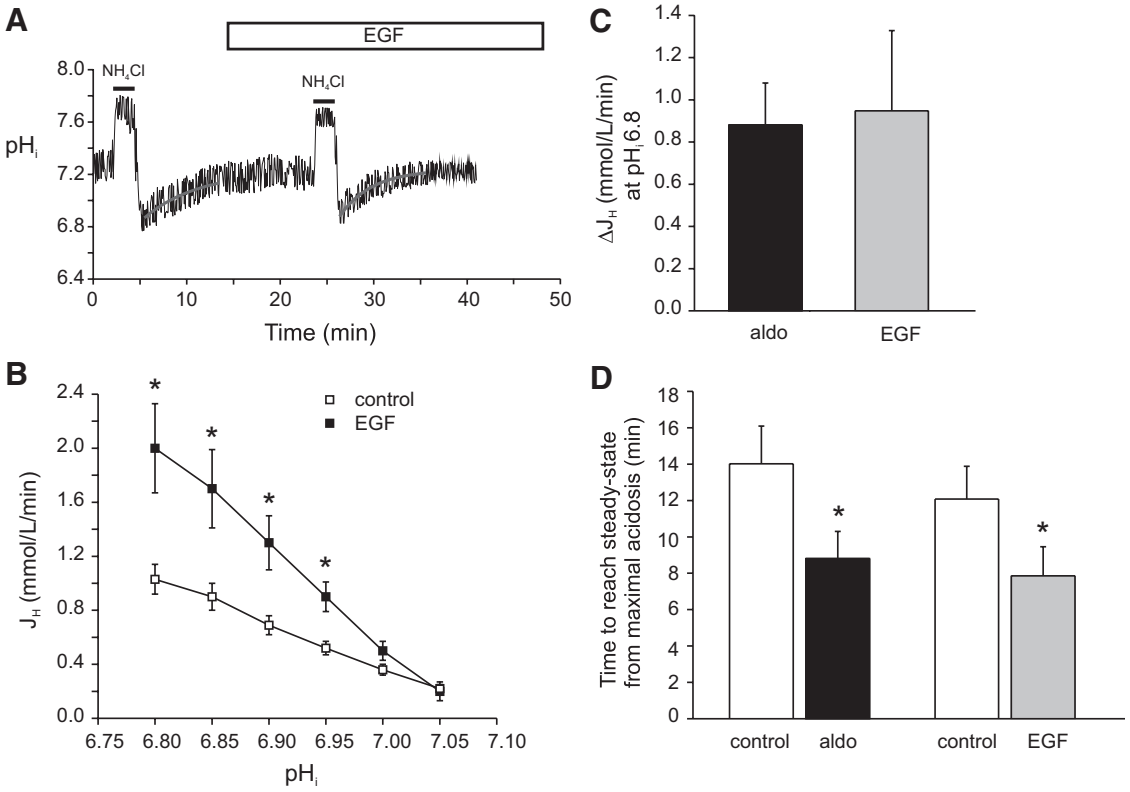

Figure 3. Epidermal growth factor (EGF) induced activation of the Na^+/H^+ exchanger (NHE-1). **A**, Representative traces of intracelluar pH (pH_i) during the application of 2 consecutive ammonium pulses (20 mmol/L of NH_4Cl), in the absence (first pulse) and presence of 0.1 μg/mL of EGF (second pulse). EGF was applied 10 minutes before the second pulse. **B**, Average proton efflux (J_H) carried by the NHE-1 before (first pulses; □; n=5) and after application of 0.1 μg/mL of EGF (second pulses; ■; n=5). *$P<0.05$ vs control. **C**, Average increase in J_H at pH_i 6.8 expressed as the difference between the second and the first pulse, in the presence of aldo (n=5) or EGF (n=5). **D**, Average time to reach steady-state pH values from maximal acidosis in the absence and presence of aldo (n=5) or EGF (n=5). *$P<0.05$ vs control.

significantly increased J_H to a similar extent to that observed with aldosterone, an effect that was prevented by AG1478. As shown in Figure 3C and 3D, the average magnitude of the effect at acidic pH and the average time to full recovery from maximal acidosis with aldosterone or EGF were similar, further supporting the idea that EGFR activation is mediating the stimulation of NHE-1 induced by aldosterone.

Role of $p90^{RSK}$ in EGFR Transactivation by Aldosterone

To demonstrate that aldosterone is able to activate the ERK 1/2-$p90^{RSK}$ cascade through the EGFR transactivation under our conditions, we determined the phosphorylation state of $p90^{RSK}$ in rat ventricular myocardium pretreated with aldosterone in the absence and presence of AG1478 or matrix metalloproteinase inhibitor II. As shown in Figure 4A, aldosterone significantly enhanced $p90^{RSK}$ phosphorylation level, an effect that was canceled by both the EGFR and the metalloproteinases inhibitors.

Considering that $p90^{RSK}$ phosphorylates the cytosolic tail of the exchanger, we decided to explore NHE-1 phosphorylation by a phosphospecific antibody, which recognizes the phospho-Ser703 in the 14-3-3 protein binding motif of the NHE-1 carboxyl tail. Aldosterone significantly increased NHE-1 phosphorylation at Ser703, and pretreatment with AG1478 or matrix metalloproteinase inhibitor II prevented this effect (Figure 4B).

We next analyzed the phosphorylation state of NHE-1 Ser703 after the myocardium pretreatment with exogenous EGF. As expected, EGF increased NHE-1 phosphorylation, an effect that was prevented by AG1478 (Figure S1, available in the online Data Supplement).

Role of Reactive Oxygen Species in EGFR Transactivation by Aldosterone

It has been demonstrated that both aldosterone[30–33] and EGF[34] stimulate reactive oxygen species (ROS) production. To test that aldosterone or EGF is also inducing the formation of ROS in rat myocardium, we measured superoxide anion by the lucigenin method. Figure S2 depicts the increase in superoxide anion generation after aldosterone (Figure S2A) or EGF (Figure S2B) treatment and the inhibition of these effects by AG1478, indicating that aldosterone increases the production of ROS via transactivation of EGFR, consistent with the effects of this hormone on NHE-1 phosphorylation and stimulation. As expected, Figure S2 also shows that spironolactone or PP1 canceled the effect of aldosterone but not that of EGF, suggesting that EGFR transactivation occurs downstream from the activation of the MR and the src-kinase and that the MR is not stimulated after EGF binding to its receptor. Spironolactone, AG1478, and PP1 did not affect the basal production of superoxide anion.

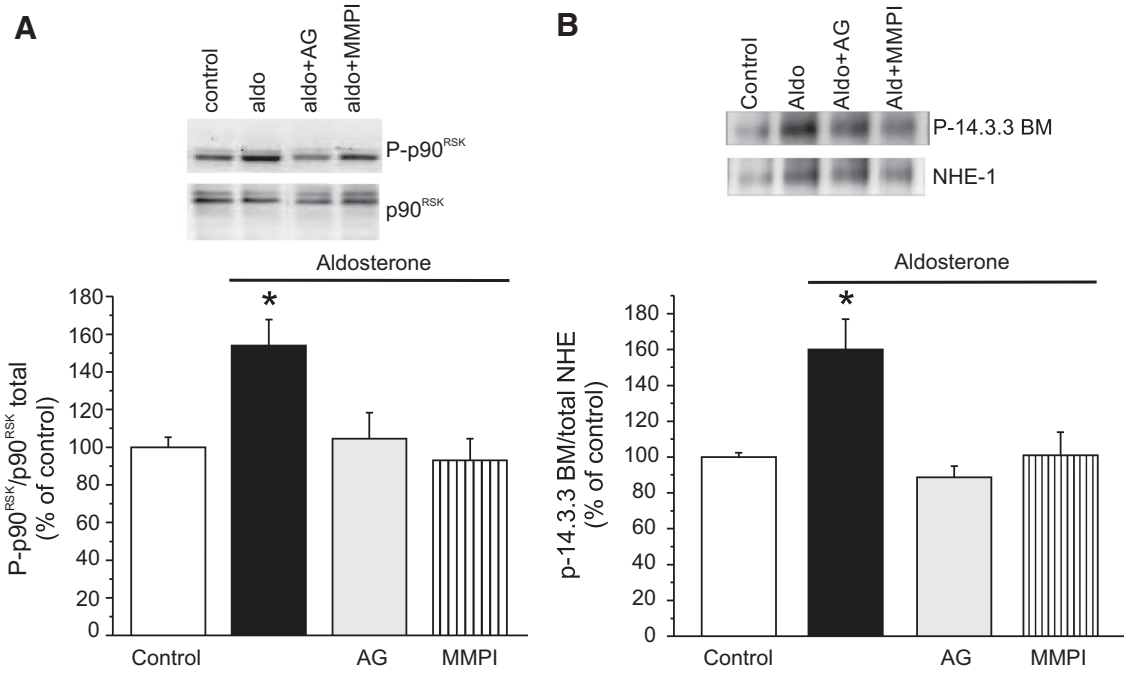

Figure 4. Aldosterone-induced p90^{RSK} and Na^+/H^+ exchanger (NHE-1) phosphorylation. **A**, p90^{RSK} activation was determined by immunoblot with a specific antibody to the phosphorylated form of the kinase (control: n=4). Aldosterone induced a significant increase in its phosphorylation (n=4), an effect that was prevented by the EGFR antagonist AG1478 (1 μmol/L; n=4) and the inhibitor of the matrix metalloproteinase inhibitor II (MMPI; 3 μmol/L; n=4). Neither AG1478 (92.01±11.51%; n=4) nor MMPI (95.50±7.98%; n=4) affected basal phosphorylation of the kinase, and no differences in total p90^{RSK} were observed between experimental groups. Representative Western blot bands are shown on top. Bars correspond with averaged ± SEM results for each group. The inhibitors and aldosterone were applied 15 and 10 minutes before freezing the ventricular slices, respectively. **B**, Aldosterone-induced activation of p90^{RSK} was accompanied by an increase in NHE-1 phosphorylation at Ser703 (n=4; control: n=4), estimated by a specific antibody against the P-14-3-3 binding motif (BM). This effect was prevented by the EGFR antagonist AG1478 (n=4) and the inhibitor of the MMPI (n=4). Again, a representative Western blot is shown on top, whereas averaged ± SEM results are expressed in the bar chart. *$P<0.05$ vs all other groups, ANOVA. The inhibitors and aldosterone were applied 15 and 10 minutes before freezing the ventricular slices, respectively.

Finally, we aimed to determine whether the ROS generated by aldosterone or EGF results in NHE-1 stimulation. As depicted in Figure S3, the effects of both hormones were abolished by pretreatment of the myocytes with the ROS scavenger *N*-2-mercaptopropionyl glycine (2 mmol/L), suggesting that ROS act as signaling molecules in the pathway triggered by transactivation of EGFR.

Discussion

Although for a long time aldosterone was exclusively seen as the most important antinatriuretic hormone exerting this effect through the intracellular MR acting as a transcription factor, early reports also called attention to the rapid nongenomic effects of this hormone.[35,36] In the present work we present for the first time that aldosterone stimulates cardiac NHE-1 through a rapid nongenomic and MR-dependent effect via the transactivation of EGFR, which, in turn, produces ROS and activates the ERK 1/2-p90^{RSK} cascade. Nongenomic but MR-independent activation of NHE-1 induced by aldosterone was described previously in the rat heart.[10,11] On the other hand, and in agreement with our data, Michea et al[37] determined that this nongenomic effect of the hormone was mediated by its binding to the MR. More recently, it was reported in vascular smooth muscle that certain nongenomic effects of aldosterone were attributed to simultaneous activation of MR and a surface membrane G protein–coupled receptor, the GPR30.[38] These investigators also reported that the MR pharmacological inhibitors used by us behave as partial antagonists of the GPR30.[38] Further research would be necessary to elucidate the potential involvement of this type of receptor in our experimental conditions.

A similar signaling pathway to the one described by us was also proposed by Downey's group[39–41] to explain Akt-dependent mitochondrial ROS production after EGFR activation. More interestingly, Gekle et al,[42] working with cultured Madin-Darby canine kidney cells, described the activation of the ERK 1/2 intracellular pathway after aldosterone-induced EGFR transactivation. These authors also suggested the NHE-1 as one of the end point targets of this mechanism present in the Madin-Darby canine kidney cells,[42] in agreement with the results obtained in cardiac myocytes presented herein.

Increasing body of evidence demonstrate that ROS also act as physiological signaling molecules of diverse intracellular pathways.[43] Moreover, cumulative data indicate that oxidative stress plays a key role in the development of cardiac hypertrophy and failure. It was demonstrated previously that exposure of rat myocytes to exogenous hydrogen peroxide leads to NHE-1 stimulation after activation of mitogen-activated protein kinase signaling.[25,44] One of these studies also involved the EGFR in this effect.[25] In cat ventricular myocytes, we have described a singular mechanism that involves

an autocrine action of endothelin 1 released from the myocyte by angiotensin II that presents the NHE-1 as an end point target of ROS acting as second messengers.[27,45] More recently, another study from our group showed that mitochondrial ROS are involved in NHE-1 stimulation produced by EGFR transactivation triggered by stretching cat papillary muscles.[34] In agreement with these studies, we suggest herein that transactivation of EGFR induced by aldosterone also involves ROS as mediators of NHE-1 stimulation. Although we did not aim to investigate the source of ROS in the present study, based on our previous work we can speculate that they are being released from mitochondria.

Pharmacological inhibition of the src-kinase or the metalloproteinases canceled the effect of aldosterone on NHE-1 stimulation. The src-kinase has been postulated previously as a link between MR activation and EGFR transactivation[17] and between G-coupled receptor activation and metalloproteinase stimulation.[34,46] We have also demonstrated that blockade of the src-kinase inhibited ROS production induced by aldosterone but not by EGF. Accordingly, Grossmann et al,[47] working with Chinese hamster ovary cells transfected with the MR, reported that ERK 1/2 phosphorylation induced by aldosterone but not that induced by EGF was inhibited by src-kinase blockade. Taken together, these data suggest that the src-kinase is the most likely candidate to be the link between the MR and metalloproteinase activation.

The results of the present work suggest for the first time that some deleterious cardiovascular effects of aldosterone could in fact be mediated by EGFR transactivation. Several studies have shown a close relationship between EGFR activation and cardiac hypertrophy. Neonatal and adult cardiomyocytes respond to both neurohumoral and mechanical growth stimuli with a marked increase in HB-EGF mRNA, which act as an early response gene to facilitate hypertrophic growth in these cells.[48] Consistently, overexpressed HB-EGF significantly induced hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts.[49] Moreover, upregulated HB-EGF exacerbated remodeling at the subacute and chronic stages postmyocardial infarction, elevating the levels of apoptosis, fibrosis, and the accumulation of myofibroblasts and macrophages, in addition to inducing left ventricular hypertrophy.[49] Zhai et al[50] demonstrated that mutations in a conserved YIPP motif in the C terminus of angiotensin II type 1 receptors, which is essential for EGFR transactivation, diminished this process and inhibited cardiac hypertrophy. More recently, it has been shown that the flavonoid silibinin has the potential to protect against cardiac hypertrophy, inflammation, and fibrosis by blocking EGFR activity and EGFR-dependent different intracellular signaling pathways.[21] Future investigation is necessary to elucidate whether these pathological effects of EGFR activation are in fact triggered by MR stimulation.

Perspectives

In the last years, the employment of new methods for screening hyperaldosteronism has revealed an important prevalence of this disease among the hypertensive patients.[51] This disease carries significant deleterious effects in heart and blood vessels, including myocardial fibrosis and endothelial dysfunction.[51,52] These patients also present higher left ventricular hypertrophy than essential hypertensive subjects.[53] In connection with this, clinical trials like the Randomized Aldactone Evaluation Study, Eplerenone Postacute Myocardial Infarction Heart Failure Efficacy and Survival Study, and, more recently, the Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure,[4] show the unequivocal beneficial effects of aldosterone inhibition in cardiac failure. The addition of an MR antagonist to the regular therapy reduced mortality by 30% in patients with cardiac failure and by 15% in patients with left ventricular dysfunction after acute myocardial infarction, respectively.[1–3] The mechanism of action by which aldosterone inhibition improves cardiac failure is not perfectly defined.[54] Because Na^+ and Ca^{2+} overload triggered by NHE-1 upregulation seems to be a well-accepted cause of myocardial hypertrophy and heart failure,[55–58] and recent studies suggest that activation of the NHE-1 is sufficient to generate Ca^{2+} signals that induce cardiac hypertrophy and heart failure,[56,59] the results obtained in the present study offer an explanation for the successfulness of MR inhibition in these circumstances. We could speculate, based in the evidence quoted before, that the decrease in ROS formation and the "deactivation" of the NHE-1 may be main contributors to the beneficial effects of aldosterone inhibition. We can also speculate that not only NHE-1 inhibition but also the inhibition of the upstream signals, like metalloproteinases, HB-EGF, and EGFR, would be beneficial interventions in myocardial hypertrophy and/or heart failure.

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Disclosures

None.

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