Aldosterone Increases NHE-1 Expression and Induces NHE-1–Dependent Hypertrophy in Neonatal Rat Ventricular Myocytes

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Abstract—We determined the effect of 24-hour aldosterone (100 nmol/L) treatment on hypertrophic responses in rat neonatal ventricular myocytes and the possible role of Na^+-H^+ exchange isoform 1 (NHE-1). Aldosterone significantly increased cell size by 61% and expression of atrial natriuretic peptide by 2-fold. NHE-1 mRNA expression and protein abundance were significantly increased, and intracellular Na^+ levels were elevated. Both hypertrophy and elevated Na^+ levels were prevented by the NHE-1–specific inhibitor EMD87580 as well as the aldosterone antagonist spironolactone, although the increased NHE-1 levels were prevented only by spironolactone. Aldosterone transiently (within 5 minutes) stimulated p44/42 phosphorylation, which decreased thereafter for the remaining 24 hours, whereas p38 phosphorylation was reduced. Neither a p38 nor a p44/42 inhibitor had any effect on aldosterone-induced hypertrophy or NHE-1 regulation. Our results therefore demonstrate a direct hypertrophic effect of aldosterone on cultured myocytes, which is dependent on NHE-1 activity. (*Hypertension*. 2003;42:1171-1176.)

Key Words: aldosterone ■ sodium-proton exchange ■ kinase ■ rats ■ hypertrophy, cardiac ■ myocytes

Cardiac hypertrophy represents a major component of myocardial remodeling contributing to heart failure.[1] Hypertrophy is mediated by endocrine, paracrine, and autocrine growth factors acting via complex cell-signaling processes.[2,3] Aldosterone might contribute to heart failure independently of its renal effects (reviewed in Slight et al[4]). Aldosterone is produced in cardiac tissue, and aldosterone receptors have been identified in the cardiac cell.[5,6] The aldosterone antagonist spironolactone has been shown to reduce mortality in patients with severe heart failure, although the precise mechanism is unknown.[7] Aldosterone infusion into uninephrectomized rats produces cardiac hypertrophy, which is attenuated by the angiotensin II type 1 receptor antagonist losartan as well by 2 calcineurin inhibitors.[8] Moreover, aldosterone failed to directly produce hypertrophy in cultured neonatal rat ventricular myocytes, although a synergistic hypertrophic effect with endothelin-1 was reported.[9]

The preceding 2 studies suggest an indirect cardiac hypertrophic influence of aldosterone. However, in view of the demonstration of cardiac aldosterone receptors,[5,6] we hypothesized that direct aldosterone receptor–mediated actions on the cardiac cell could be predicted and therefore, determined whether it exerts direct hypertrophic effects. We focused on the potential role of the Na^+-H^+ exchanger isoform 1 (NHE-1) in this response, because inhibition of the antiporter has been shown to attenuate myocardial hypertrophy and heart failure.[10–13] Moreover, aldosterone activates NHE-1, including that in cardiac cells,[14,15] although with respect to the latter, this occurred only after 9 days of treatment.[15] Patients with primary aldosteronism have increased erythrocyte NHE activity.[16] In the present study, we examined the direct effects of the mineralocorticoid on cell hypertrophy and the potential contribution of NHE-1 to this process.

Methods

Primary Cultures of Isolated Neonatal Myocytes

Primary myocyte cultures were prepared from neonatal Harlan Sprague-Dawley rat heart ventricles as described previously[17] and in accordance with guidelines of the Canadian Council on Animal Care. Myocytes were first maintained for 48 hours in Dulbecco's Eagle's/ Ham's F-12 modified medium.

Experimental Protocol

Cells were serum-starved for 24 hours, after which they were treated for 24 hours with 100 nmol/L aldosterone (Sigma). This represents the lowest concentration required to obtain a maximum increase in cell size, as determined from initial concentration-response studies. Moreover, 48-hour exposure to 100 nmol/L aldosterone did not produce significantly greater effects than those observed with 24-hour treatment. For some experiments, cells were first pretreated with the NHE-1 isoform–specific inhibitor EMD87580 (5 μmol/L, Merck KGaA), the aldosterone antagonist spironolactone (1 μmol/ L), the p44/42 inhibitor PD98059 (100 μmol/L), or the p38 inhibitor SB203580 (100 μmol/L), all from Sigma, 15 minutes before aldosterone addition.

Measurement of Cell Surface Area

Cardiomyocyte surface area was determined for 50 randomly selected cells per experiment and averaged to provide an N value of 1. Cells were visualized with a Leica inverted microscope equipped with a Polaroid digital camera under $20\times$ magnification, and cell area was determined with the use of Mocha software (MochaSoft Aps).[18]

Competitive RT-PCR for Estimation of NHE-1 and ANP Gene Expression

Reverse transcription–polymerase chain reaction (RT-PCR) was used to analyze NHE-1 and atrial natriuretic peptide (ANP) mRNA expression as described previously.[19] The NHE-1 primers used were primer 1 [(+), 5′-TCTGTGGACCTGGTGAATGA-3′] and primer 2 [(−), 5′-GTCACTGAGGCAGGGTTGTA-3], with a predicted product size of 210 bp and a competitor size of 292 bp. The forward and reverse primers for rat ANP were 5′-CTGCTAGACCACCTGGAGGA-3′ and 5′-AAGCTGTTGCAGCCTAGTCC-3′, respectively, with a PCR product of 320 bp. Samples were then electrophoresed in 1.5% agarose gels containing ethidium bromide and quantified by densitometry.

Western Immunoblotting

NHE-1 protein was measured with a anti–NHE-1 monoclonal antibody (Chemicon) as described earlier.[19] Antibodies for detection and quantification of extracellular signal–regulated kinase ([ERK] p44/42), phosphorylated ERK (pERK), p38, and phosphorylated p38 (pp38) were from Santa Cruz. Immunoblotting and subsequent analysis were performed as described previously.[17]

Confocal Microscopy

Cytosolic and nuclear sodium levels were determined by confocal microscopy as described previously.[20] Sodium green (Molecular Probes)–loaded cells were examined with a Molecular Dynamics Multi Probe 2001 confocal argon laser scanning system equipped with a Nikon Diaphot epifluorescence inverted microscope and a $60\times$ Nikon Oil Plan achromat objective. The pinhole size was set at 100 μm, and the image size was set at 512×512 pixels with a pixel size of 0.34 μm. At the end of each experiment, the nucleus was stained with the nucleic acid stain Syto 11. Measurements of cytosolic and nuclear sodium were performed from 3-dimensional reconstructions of the cell, including the nucleus, as previously described.[20] Sodium levels are represented as mean fluorescence intensity values.

Statistics Analysis

Results are given as mean±SEM. Statistical analyses were performed by 1-way ANOVA. The posttest comparison was performed by the method of Bonferroni. Differences were considered significant with $P<0.05$.

Results

Effect of Aldosterone on Myocyte Hypertrophy

Aldosterone produced a significant increase ($\approx$61%) in cell surface area and a concomitant 2-fold increase in ANP expression. These were completely prevented by both spironolactone and EMD87580, neither of which exerted direct effects on its own (Figure 1).

Effect of Aldosterone on NHE-1 mRNA and Protein Expression

As shown in Figure 2, NHE-1 mRNA expression was doubled in aldosterone-treated myocytes ($P<0.05$). This was unaffected by EMD87580 but completely prevented by spironolactone. Figure 3 shows representative examples of Western blots (A) for NHE-1 of cardiac myocytes treated for 24 hours with aldosterone and a monoclonal anti–NHE-1
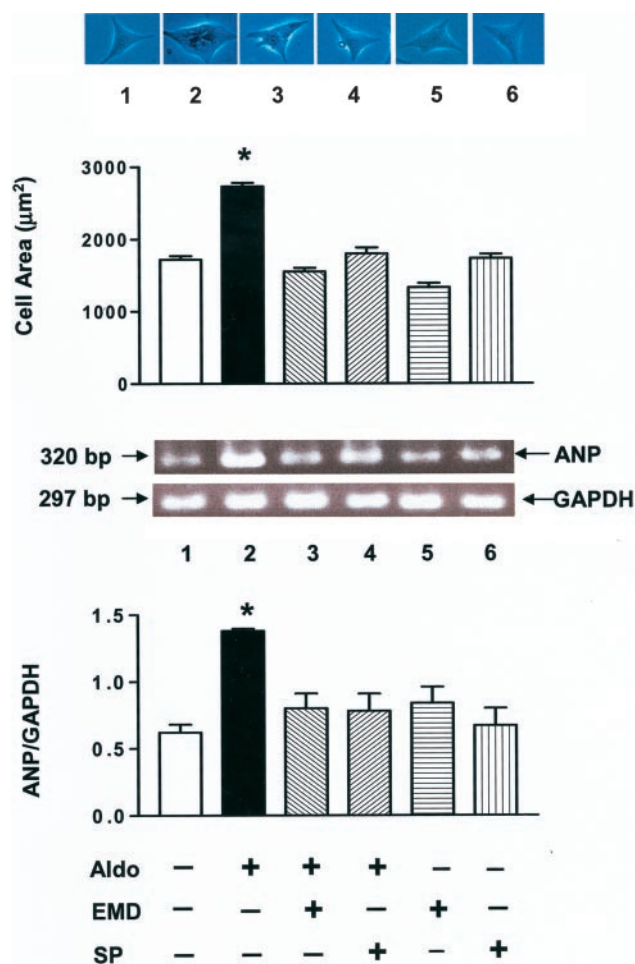

Figure 1. Effect of 24-hour aldosterone (Aldo) treatment on cell surface area and ANP expression in cultured neonatal rat ventricular myocytes and influence of the NHE-1 inhibitor EMD87580 (EMD) and the aldosterone antagonist spironolactone (SP). Treatment groups were as follows: 1, untreated; 2, Aldo alone; 3, Aldo plus EMD; 4, Aldo plus SP; 5, EMD alone; and 6, SP alone. Values are mean±SEM, n=10 for each treatment group. Top figures demonstrate representative photomicrographs of myocytes after treatments, whereas middle figure shows agarose gels of RT-PCR products for ANP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *$P<0.05$ compared with all other groups. Drug concentrations were as described in Methods.

antibody. Panel B demonstrates quantitative assessment and shows a significant increase in NHE-1 protein. This was completely blocked by spironolactone (not shown).

Effect of Aldosterone on Mitogen-Activated Protein Kinases (MAPKs)

Figure 4A shows representative Western blots for pp44/42 at 5, 10, 15, and 20 minutes after aldosterone treatment, whereas Figure 4B illustrates 24-hour-treatment results. Figure 4 (C and D) demonstrates quantitative assessments for pp44/42 and total p44/42, respectively. Aldosterone significantly increased pp44/42 levels by 5 minutes, which rapidly declined to values significantly below that of control (Figure 4C, left) and that persisted for 24 hours (Figure 4C, right). Total p44/42 protein was unaffected by short-term treatment (Fig-
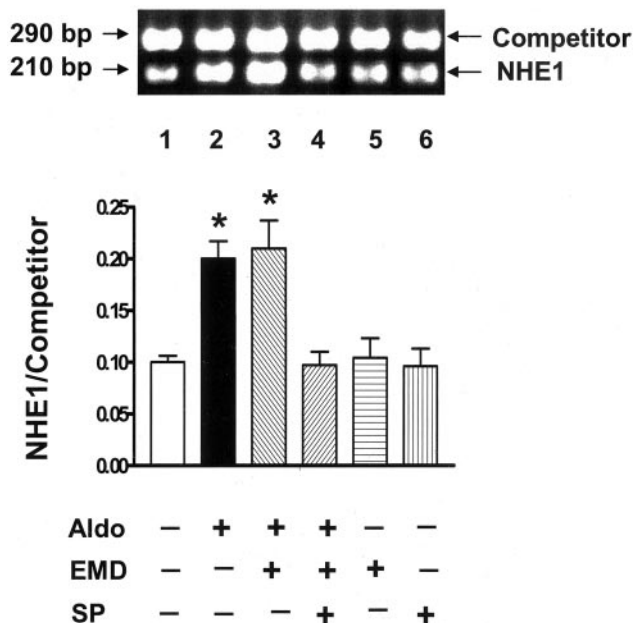
Figure 2. RT-PCR analysis of NHE-1 expression in myocytes after various treatments. Top, Example of ethidium bromide–stained gels of RT-PCR products according to the following treatments: 1, untreated; 2, Aldo; 3, Aldo plus EMD; 4, Aldo plus SP; 5, EMD alone; and 6, SP alone. Data were quantified by densitometry and are presented as the ratio of competitor to NHE1. Values are mean±SEM, n=6 for each treatment group. Abbreviations are defined in the legend to Figure 1. *$P<0.05$ compared with other groups.

ure 4D, left), although a variable increase in levels was noted after 24-hour treatment, which was not statistically significant (Figure 4D, right).

Figures 5A and 5B show that treatment with aldosterone reduced the level of pp38. Stimulation with aldosterone
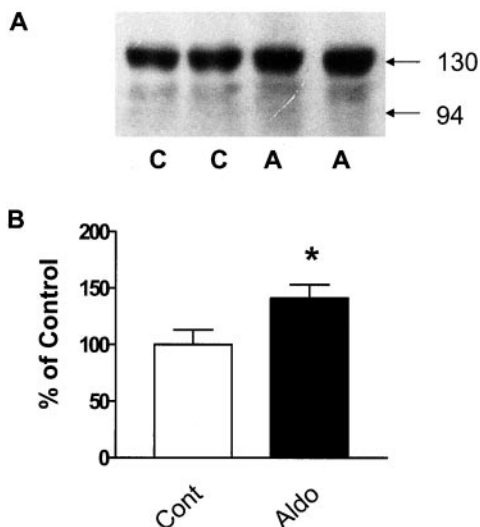

Figure 3. Effects of 24-hour aldosterone (Aldo) treatment on NHE-1 protein levels in myocytes. A, Example of Western blots of control (Cont, C) and aldosterone-treated (A) cells. B, Summary of effects of aldosterone treatment on Na$^+$-H$^+$ exchanger levels of aldosterone-treated isolated myocytes. Values are mean±SEM, n=20 for control and 28 for aldosterone group. *$P<0.05$ from control.
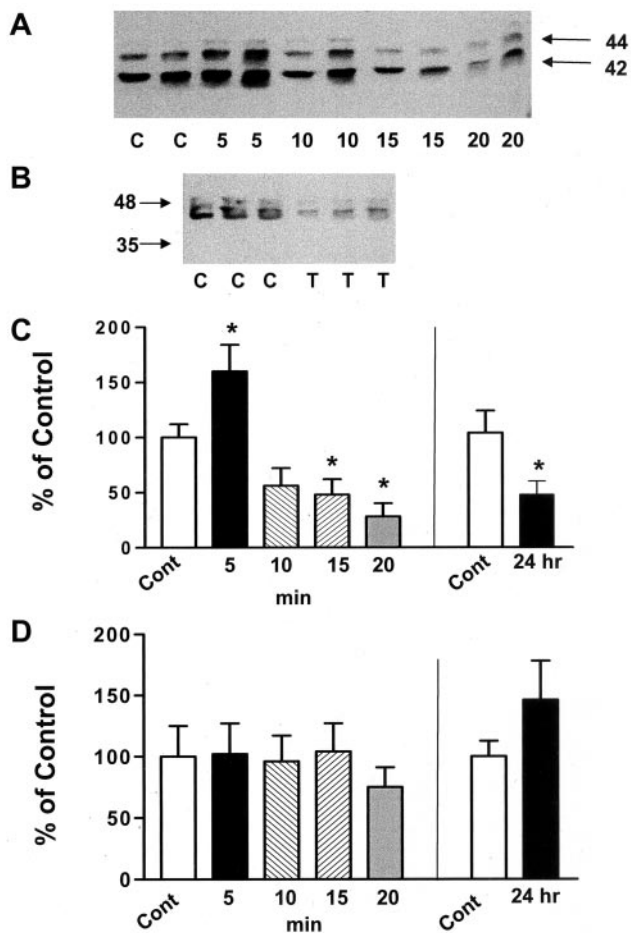

Figure 4. Effect of aldosterone treatment on ERK (p44/42) in myocytes. A, Example of effects of short-term aldosterone treatment on pp44/42 levels by Western blotting. Myocytes were treated with aldosterone for 5, 10, 15, or 20 minutes. B, Example of effect of long-term aldosterone treatment on pp44/42 levels. Myocytes were treated with aldosterone for 24 hours, and pp44/42 was examined, as in Figure 4A. C indicates controls matched for treatment with vehicle alone; T, 24-hour aldosterone–treated isolated myocytes. C, Summary of effects of aldosterone treatments on pp44/42 levels. Left, Effect of 5-, 10-, 15-, or 20-minute treatment with aldosterone. Right, Effect of 24-hour treatment with aldosterone. *$P<0.05$ from controls, n≥10 for all determinations. D, Effect of aldosterone treatment on ERK protein levels in isolated myocytes treated with aldosterone. Myocytes were treated with aldosterone for 5 to 20 minutes or for 24 hours, as described for Figure 4A or 4B. Both ERK1 (p44) and ERK2 (p42) immunoreactive bands were included for analysis. Results are a summary of at least 5 experiments. Cont indicates control.

caused a significant decline in the level of p38 after 10 minutes (20% to 25%), and this increased approximately to a 30% decline after 20 minutes. Neither total p38 nor pp38 levels were affected after 24-hour aldosterone treatment (Figures 5C through 5E).

Effect of MAPK Inhibition

Figure 6 shows that neither the p44/42 inhibitor PD98059 nor the p38 inhibitor SB203580 had any effect on aldosterone-stimulated NHE-1 expression in cardiomyocytes either individually or when administered in combination. Moreover, as
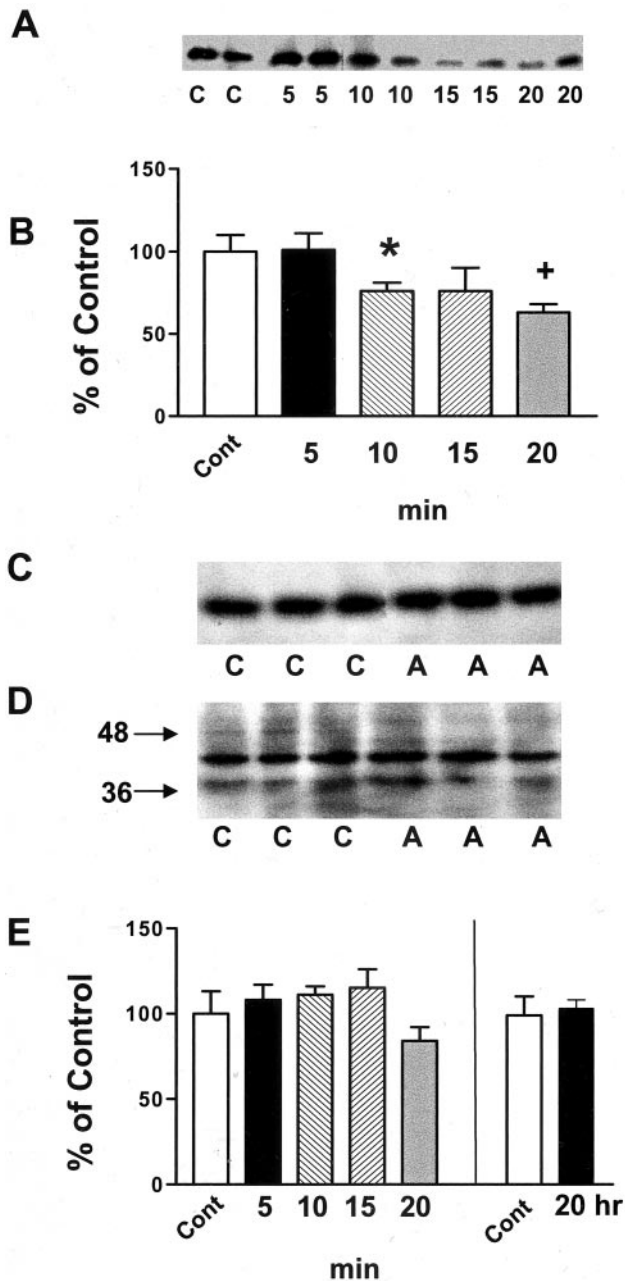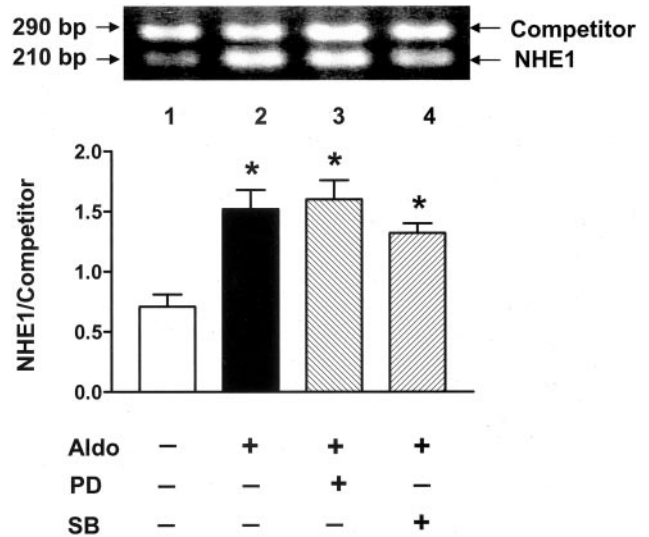
Figure 6. RT-PCR analysis of NHE-1 expression in myocytes after various treatments and the effect of MAPK inhibitors. Top, Example of ethidium bromide–stained gels of RT-PCR products according to the following treatments: 1, untreated; 2, Aldosterone (Aldo); 3, Aldo plus the p44/42 inhibitor PD98059 (PD); and 4, Aldo plus the p38 inhibitor SB203580 (SB). Data were quantified by densitometry and are presented as the ratio of competitor to NHE-1. Values are mean±SEM, n=7 for each treatment group. *$P<0.05$ compared with untreated group.

Figure 5. Effect of aldosterone treatment on p38 in myocytes. A, Examples of a Western blot of isolated cardiac myocyte extracts with anti-pp38 antibody. Myocytes were treated with aldosterone for 5, 10, 15, or 20 minutes. B, Summary of effects of aldosterone treatment on pp38 levels of isolated cardiac myocytes. C, Effects of 24-hour aldosterone treatment on protein levels of p38. D, Example of effects of 24-hour aldosterone treatment on pp38 levels. C or Cont indicates control; A, aldosterone treated. E, Summary of effects of aldosterone treatment on p38 protein levels. n≥6 for all groups.

demonstrated in Figure 7, cardiomyocyte hypertrophy was unaffected by any of the agents.

Potential Role of Na$^+$
As shown in Figure 8, aldosterone produced a marked elevation in intracellular Na$^+$ levels, although this was restricted to apparent intracellular pools or clusters and was distinct from nuclei imaged with Syto 11.

Discussion
The aldosterone receptor antagonist spironolactone has been shown to improve survival in heart failure patients,[7] and aldosterone levels are upregulated in heart failure, even under angiotensin-converting enzyme inhibition.[21] How aldosterone contributes to heart failure is not known with certainty. Aldosterone might contribute to remodeling by stimulation of extracellular matrix (reviewed in Zannad et al[22]), and a significant relation between aldosterone levels and left ventricular hypertrophy has been demonstrated.[23] Although evidence for a direct hypertrophic effect of aldosterone on the cardiomyocyte is lacking, 6-week aldosterone administration to uninephrectomized rats maintained on a high-sodium diet resulted in marked myocardial hypertrophy as well as blood pressure elevation.[8] Interestingly, left ventricular hypertrophy and elevated plasma aldosterone levels in hypertensive patients were associated with increased erythrocyte NHE activity,[24] and both cariporide and spironolactone have been found to reduce cardiac fibrosis in uninephrectomized rats given an 8-day mineralocorticoid-salt treatment, thus suggesting a link between aldosterone and NHE-1 in producing fibrosis in this model.[25] Accordingly, we focused our study on the potential role of NHE-1 in mediating aldosterone's effects.

Our study demonstrates that aldosterone can directly produce cardiomyocyte hypertrophy via an NHE-1–dependent pathway. Indeed, the ability of the NHE-1 inhibitor EMD87580 to prevent the hypertrophy was virtually identical to that observed with spironolactone. EMD87580 did not affect the aldosterone-induced increase in NHE-1 expression, a finding in apparent contrast to in vivo studies wherein treatment with NHE-1–specific inhibitors attenuated the upregulated NHE-1 expression in the hypertrophied myocardium in isoproterenol-treated rats[26]
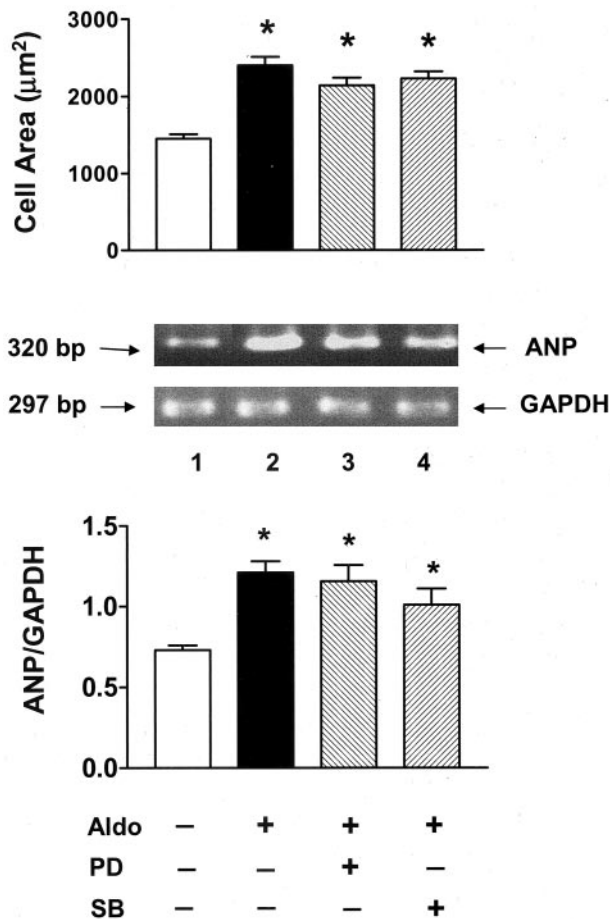
Figure 7. Effects of MAPK inhibitors on aldosterone-induced hypertrophic response. A, Myocyte surface areas under different treatments. B, ANP expression analyzed by RT-PCR according to the following treatments: 1, untreated; 2, Aldosterone (Aldo); 3, Aldo plus the p44/42 inhibitor PD98059 (PD); and 4, Aldo plus the p38 inhibitor SB203580 (SB). Data were quantified by densitometry and are presented as the ratio of competitor to NHE-1. Values are mean±SEM, n=7 for each treatment group. *$P<0.05$ compared with untreated group.
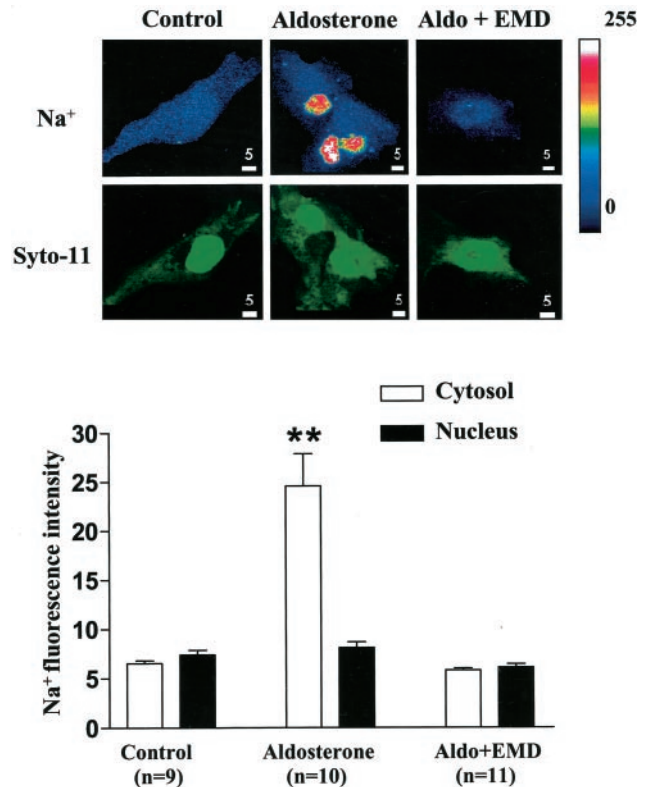

Figure 8. Top, Confocal images demonstrating Na$^+$ green fluorescence (top) and nuclear staining with Syto-11 (bottom) in control cardiomyocytes and in the presence of aldosterone with or without EMD87580 (EMD). Bottom, Quantitative assessment of Na$^+$ fluorescence intensity in cytosol and nucleus with these treatments. **$P<0.01$ compared with control and Aldo+EMD groups.

or in mice overexpressing the β_1-adrenoceptor.[13] Moreover, the upregulation in NHE-1 expression in the acutely ischemic and reperfused myocardium was found to be attenuated by the NHE-1–specific inhibitor cariporide.[27] In contrast, 1-month administration of the NHE-1–specific inhibitor cariporide to normal rats produced an 80% increase in myocardial NHE-1 expression.[28] Thus, the response to NHE-1 inhibitors in terms of NHE-1 expression appears to be diverse and possibly dependent on the experimental model or the nature of the initial stimulus to upregulate NHE-1.

Aldosterone induced a marked elevation in intracellular Na$^+$ levels, which was confined to distinct pools and not widely distributed within the myocyte, and which was completely prevented by NHE-1 inhibition. The locus of the Na$^+$ pooling within the cell remains uncertain but might be related to the fact that the distribution of NHE-1 is not uniform but concentrated in discrete regions of the cell membrane.[29] Moreover, intracellular Na$^+$ and H$^+$ are not always freely diffusible and can remain concentrated in discrete subsarcolemmal regions for periods of time.[30,31] A role for Na$^+$ in mediating NHE-1–dependent car-

diomyocyte hypertrophy has been previously proposed, suggesting Na$^+$-induced protein kinase C (PKC) activation as mediating these effects.[32] However, aldosterone has been shown to inhibit PKC,[33] and we have been unable to observe any modulatory role of the PKC inhibitor bisindolylmalemide on aldosterone-induced hypertrophy (data not shown).

MAPK is an important regulator of NHE-1 activity as well as cardiac hypertrophy,[17,34,35] and aldosterone has been shown to activate NHE in some cells via a p44/42-dependent pathway.[14] Aldosterone produced a rapid albeit transient activation of p44/42 followed by a sustained reduction in phosphorylation, a phenomenon similar to our previous observation where serum was the activating factor.[34] In the case of p38, longer treatments with aldosterone caused a decrease in the level of pp38, independent of changes in protein levels, although as for p44/42, the specific mechanisms underlying these effects require further studies. Neither p38 nor p44/42 inhibition influenced any response to aldosterone.

It should be noted that our finding of a direct hypertrophic effect of aldosterone on cardiomyocytes differs from a recent report that failed to demonstrate a direct hypertrophic effect of 1 μmol/L aldosterone after 15-hour treatment in rat neonatal cardiac myocytes.[9] However, aldosterone did augment the hypertrophic effect of endothelin-1, thus demonstrating a synergistic relation.[9] The reason for this apparent discrepancy needs to be resolved but might reflect the large difference in aldosterone concentrations that were used.

Accordingly, we used a 10-fold lower dose of aldosterone, which is a closer approximation to the in vivo situation, particularly under hyperaldosteronemic conditions.

Perspectives

The present study demonstrates a direct hypertrophic effect of aldosterone in cultured neonatal rat ventricular myocytes. This effect was associated with increased protein levels of NHE-1 and was dependent on NHE-1 activity. The precise signaling pathways linking aldosterone to NHE-1 require elucidation but appear not to involve MAPK. The results should be interpreted cautiously in view of potential differences, such as in cell-signaling mechanisms or aldosterone receptor density, which might exist between neonatal and adult myocytes. Moreover, the concentration of aldosterone used in this study was supraphysiologic, which was done to produce maximum consistent effects with a relatively brief treatment duration. Our findings suggest a direct effect of aldosterone on cardiomyocyte hypertrophy via an NHE-1–dependent pathway. Taken together, it is likely that aldosterone exerts its effects via multiple sites, both cardiac and extracardiac, through which it contributes to myocardial remodeling.

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