Na/H Exchange Isoform 1 Is Involved in Mineralocorticoid/Salt-Induced Cardiac Injury

Genro Fujisawa, Koji Okada, Shigeaki Muto, Nobuya Fujita, Naoki Itabashi, Eiji Kusano, Shun Ishibashi

Abstract—Long-term exposure of uninephrectomized rats to desoxycorticosterone acetate (DOCA)/salt induces cardiac fibrosis and hypertrophy through mineralocorticoid receptors (MRs). However, the underlying cellular mechanisms remain unclear. To determine whether Na/H exchange isoform 1 (NHE1) is involved in the cellular mechanisms, we examined the effects of a specific NHE1 inhibitor, cariporide, and an MR antagonist, spironolactone, on DOCA/salt-induced cardiac fibrosis and hypertrophy. Uninephrectomized rats were given 20 mg of DOCA (single subcutaneous injection) plus 0.9% NaCl/0.3% KCl to drink and were killed at 8 days. Two groups of rats given DOCA/salt were treated with either spironolactone (50 mg/kg per day SC) or cariporide (30 mg/kg per day PO) for 8 days. Control rats were treated with only high salt after the operation. The DOCA/salt-induced perivascular collagen deposition was completely abolished by cariporide and spironolactone. DOCA/salt-induced interstitial collagen deposition was partially and completely suppressed by spironolactone and cariporide, respectively. The rats exposed to DOCA/salt had cardiocyte hypertrophy in the subendocardial and subepicardial regions, a finding that was completely inhibited by cariporide but not by spironolactone. In rats given DOCA/salt, NHE1 protein expression was markedly increased. This was partially and completely reversed by spironolactone and cariporide, respectively. We concluded that cardiac NHE1 contributes to DOCA/salt-induced cardiac fibrosis and hypertrophy and that the NHE1 inhibitor cariporide completely prevents the detrimental effects of DOCA/salt on the heart. We also demonstrated that DOCA/salt-induced cardiac injury through the MRs partly occurs through NHE1 activation. (Hypertension. 2003;41:493-498.)

Key Words: mineralocorticoids ■ fibrosis ■ hypertrophy ■ collagen ■ sodium-hydrogen exchanger

Several lines of evidence indicate that long-term treatment of uninephrectomized (UNX) rats with mineralocorticoid/salt results in arterial hypertension, cardiac hypertrophy, and cardiac fibrosis.1-4 The effect of mineralocorticoid/salt on cardiac fibrosis was blocked by treatment with spironolactone, the mineralocorticoid receptor (MR) antagonist.3 However, it is not known how the exposure to mineralocorticoid/salt induces cardiac fibrosis through MR.

The Na/H exchange isoform 1 (NHE1) is a plasma membrane transport protein found in cardiovascular cells.5,6 In cardiocytes, activation of the NHE1 results in increases in intracellular Na+ and/or intracellular pH (pH) and consequently may mediate the inotropic responses of the myocardium to neurohormonal stimuli7,8 and myocardial stretch.9 Sarcolemmal NHE activity may also play a permissive role in the hypertrophic response of cardiocytes to neurohormonal10 and mechanical6 stimuli in vitro. Ebata et al11 reported that the exposure of vascular smooth muscle cells to aldosterone for 24 hours increased both NHE1 mRNA levels and NHE activity. In cultured neonatal rat cardiocytes, aldosterone was shown to activate NHE.12 The inhibitors of NHE1 were reported to have a protective effect on hearts recovering from ischemia.5,13 These reports suggest that NHE1 may be involved in mineralocorticoids/salt-induced cardiac fibrosis. However, direct evidence for the involvement of NHE1 in mineralocorticoids/salt-induced cardiac fibrosis is lacking. Spironolactone prevented cardiac collagen synthesis and fibrosis in DOCA/salt rat models5,8 and inhibited the stimulatory effect of aldosterone on NHE activity in vascular smooth muscle cells,11 suggesting that spironolactone may act by the inhibition of NHE1. Thus, the present study compared the effects of cariporide on mineralocorticoid/salt-induced cardiac fibrosis, cardiac hypertrophy, arterial hypertension, and cardiac NHE1 protein expression with those of spironolactone.

The purpose of the present study was therefore to address whether NHE1 contributes to mineralocorticoid/salt-induced cardiac fibrosis, cardiac hypertrophy, and/or hypertension. For this purpose, we chose cariporide (HOE642) as the NHE1 inhibitor because it is highly specific for the cardiac isoform of NHE1 (NHE1).5,13,14 Also, the present study was undertaken to examine whether NHE1 protein levels are increased in the
myocardium from rats given DOCA/salt, and if so, whether the increased expression of the NHE1 protein is inhibited after treatment with cariporide.

Fujisawa et al. reported time-dependent effects on blood pressure and cardiac histology for up to 32 days when UNX rats receiving 0.9% NaCl drinking water were injected weekly with 20 mg DOCA. They found that there were relatively early elevations in both blood pressure and cardiac fibrosis after 4 days of DOCA/salt, whereas levels of scar tissue increased after 16 and 32 days of DOCA/salt. As we focused on the mechanisms responsible for DOCA/salt-induced cardiac fibrosis and hypertrophy, in this study we singly injected DOCA at 20 mg to UNX rats and observed blood pressure and cardiac histology after 8 days.

Methods

Animals
All experimental protocols for this animal study were approved by the Animal Ethics Committee of Jichi Medical School. Male Sprague-Dawley rats (SLC, Tokyo, Japan) weighing 180 to 200 g were anesthetized with Brevital sodium (55 mg/200 g body wt IP; Eli Lilly), and the left kidney was removed. DOCA (Sigma) or DOCA combined with either spironolactone (Pharmacia) or cariporide (HOE642, Aventis Pharma) was given. DOCA (20 mg per rat SC) dissolved in peanut oil was given for 8 days. Cariporide (30 mg/kg body wt per day in 3 divided doses at 8-hour intervals PO) dissolved in distilled water was delivered for 8 days. Control rats were operated but were untreated with DOCA. All rats were given standard rat chow and 0.9% NaCl/0.3% KCl ad libitum for 8 days. Animals were divided into 4 groups (n=6/group): (1) control/salt plus vehicle, (2) DOCA/salt plus vehicle, (3) DOCA/salt plus spironolactone, and (4) DOCA/salt plus cariporide.

The rats were trained in the blood pressure device to accustom them to the procedure. Systolic blood pressures were measured by tail-cuff plethysmography (Softran) the day before killing. The body weights were then recorded, and animals were decapitated. The heart was then removed and weighed. The middle third portion of a ventricular tissue sample was fixed in 3.7% formaldehyde solution, and the specimens were embedded in paraffin and cut into 5-μm-thick slices transversely on a microtome.

Histology and Immunohistochemistry
The cardiac tissues were used for hematoxylin and eosin, and picrosirus red staining and immunohistochemistry of the NHE1 protein (avidin-biotin peroxidase complex method, VECTASTAIN Elite ABC kit, Vector Laboratory).

For immunohistochemistry, sections were exposed to rabbit polyclonal antibodies against NHE1 protein (dilution 1:100; Chemicon International), followed by a biotinylated goat anti-rabbit antibody (dilution 1:200; Vector Laboratories). Normal rabbit IgG was used as a negative control.

Morphometric Analysis
Sections stained with picrosirus red were analyzed for interstitial and perivascular collagen with an image analysis system. The interstitial collagen volume fraction was measured from the area of stained tissue within a given field. Twenty fields for each heart transverse section were selected at random. The area stained was calculated as a percentage of the total areas within a field (magnification ×100). The perivascular collagen fraction was measured as the area for a sample of the vessels in the section (magnification ×100). To correct for differences in vessel size, the perivascular collagen fraction was expressed as perivascular collagen area per media area.

Results
First, we measured systolic blood pressure in control rats plus vehicle and rats given DOCA/salt plus either a vehicle, spironolactone, or cariporide by tail-cuff plethysmography. Results are shown in Figure 1A. In rats given DOCA/salt, systolic blood pressure was 153 mm Hg, which was significantly greater than in control rats (119 ± 2 mm Hg). Treatment of rats given DOCA/salt with spironolactone significantly decreased the systolic blood pressure level to 115 ± 3 mm Hg, which was not significantly different from that in control rats. In sharp contrast, treatment with caripo-
ride had no effect on DOCA/salt-induced blood pressure elevation.

Next, we measured heart weight in the 4 groups of rats. As shown in Figure 1B, heart weight in the 4 groups of rats showed a different pattern of evolution in systolic blood pressure. Exposure of rats to DOCA/salt markedly increased heart weight to 3.74±0.04 mg/g body wt as compared with the control rats (3.10±0.04 mg/g body wt). Heart weight in rats treated with DOCA/salt plus spironolactone was 3.52±0.06 mg/g body wt, a value significantly lower than in rats treated with DOCA/salt but still significantly greater than in control rats. Heart weight in rats exposed to DOCA/salt plus cariporide was 3.31±0.04 mg/g body wt, significantly lower than that in rats exposed to DOCA/salt but still significantly greater than in control rats and significantly lower than in rats given DOCA/salt plus spironolactone.

Because alterations in heart weight include development of cardiac fibrosis, we next examined collagen deposits in hearts from the 4 groups of rats by observing the alteration of collagen accumulation with picrosirius red staining and then analyzing interstitial and perivascular collagen with a computer image analysis system. Figures 2A and 2B show the collagen volume fraction in interstitial and perivascular regions, respectively. The interstitial collagen volume fraction in animals exposed to DOCA/salt was 0.84±0.01%, significantly greater than in control animals (0.63±0.04%). In animals given DOCA/salt plus spironolactone, the interstitial collagen volume fraction was 0.69±0.05%, the value that was significantly lower than in animals given DOCA/salt, but was not significantly different from that in control animals. In animals given DOCA/salt plus cariporide, the interstitial collagen volume fraction was 0.57±0.07%, significantly lower than that in animals given DOCA/salt alone but not significantly different from that in control animals. The perivascular collagen volume fraction in rats treated with DOCA/salt (1.81±0.16%) was also significantly greater than in control rats (0.91±0.03%). Treatment with cariporide or spironolactone in rats given DOCA/salt completely inhibited the DOCA/salt-induced elevation of the perivascular collagen volume fraction (0.86±0.08% or 0.77±0.07%, respectively).

Because the alteration in heart weight is also involved in the development of cardiac hypertrophy, we next examined whether treatment with spironolactone or cariporide in rats given DOCA/salt influenced the size of cardiocytes by measuring cardiocyte cross-sectional areas in the subendocardial and subepicardial regions of the left ventricle from the 4 groups of rats. Cardiocyte cross-sectional areas in the subendocardial and subepicardial regions of the left ventricle in the 4 groups of rats are shown in Figures 3A and 3B, respectively. In rats exposed to DOCA/salt, the cross-sectional area in the subendocardial region was 332.2±39.6 µm², a value that was significantly greater than in control rats (243.5±11.0 µm²). In rats exposed to DOCA/salt and spironolactone, the
Cardiocyte cross-sectional area in the subendocardial region was $305.7 \pm 10.6 \mu m^2$, which was significantly greater than that in control rats but not significantly different from that in rats exposed to DOCA/salt alone. In sharp contrast, in rats exposed to DOCA/salt plus cariporide, the cardiocyte cross-sectional area in the subendocardial region was $201.5 \pm 13.4 \mu m^2$, which was significantly greater than that in rats exposed to DOCA/salt alone but was not significantly different from that in control rats. The alteration in size of the cardiocyte in the subepicardial region was similar to that in the subendocardial region. DOCA/salt administration also increased the cardiocyte cross-sectional area in the subepicardial region to $308.2 \pm 8.2 \mu m^2$ when compared with the control group ($210.4 \pm 13.3 \mu m^2$). Spironolactone ($319.0 \pm 10.9 \mu m^2$) had no effect on the DOCA/salt-induced increase in cardiocyte cross-sectional area, whereas cariporide ($181.0 \pm 4.8 \mu m^2$) completely inhibited this DOCA/salt-induced increase.

Finally, we examined whether NHE1 is actually expressed in rats given DOCA/salt with or without treatment with spironolactone and cariporide by staining the myocardium with an antibody against NHE1 protein, as shown in Figure 4. In control rats, there was little NHE1 staining in cardiocytes. On the other hand, in rats exposed to DOCA/salt alone, almost all cardiocytes of each heart section were stained with the NHE1 protein. In rats exposed to DOCA/salt plus spironolactone, almost all cardiocytes in each heart section were also stained with the NHE1 protein, but it was weaker than in rats exposed to DOCA/salt alone. In the 2 groups of rats, the stained particles were observed in whole cardiocytes. In sharp contrast, in rats exposed to DOCA/salt plus cariporide, the NHE1 staining was completely normalized.

**Discussion**

Chronic excess mineralocorticoids in the circulation combined with a high-salt diet in UNX rats have been reported to cause hypertension, cardiac hypertrophy, and fibrosis.1–4 In this model, spironolactone had an antifibrotic effect on the heart,1 a finding attributed to blockade of tissue MRs. The
The goal of the present study was to determine the mechanisms by which mineralocorticoid/salt induces cardiac fibrosis and hypertrophy via MRs, especially the involvement of NHE1 in the detrimental effects of the exposure to mineralocorticoids/salt on the heart. To do this, we used the MR antagonist spironolactone and the NHE1 inhibitor cariporide to compare the effects of the 2 drugs on DOCA/salt-induced hypertension, cardiac fibrosis, hypertrophy, and NHE1 protein expression.

We observed that in UNX rats receiving 0.9% NaCl drinking solution, 8 days after single injections of subcutaneous DOCA at 20 mg, there were significant increases in arterial blood pressure, heart weight, perivascular and interstitial cardiac fibrosis, and cardiocyte hypertrophy in the subendocardial and subepicardial regions. Interestingly, we also found that the NHE1 protein was markedly expressed in rats given DOCA/salt. Coadministration with cariporide had no effect on DOCA/salt-induced arterial hypertension but completely reversed the DOCA/salt-induced increase in heart weight, completely prevented DOCA/salt-induced interstitial and perivascular fibrosis, and completely inhibited DOCA/salt-induced cardiocyte hypertrophy in the subendocardial and subepicardial regions. The DOCA/salt-induced NHE1 protein expression was fully reversed by the treatment with cariporide. Cariporide is a highly selective NHE1 inhibitor with 60-fold selectivity over NHE2 and 3000-fold selectivity over NHE3.14 Taken together, the present findings are consistent with the notion that the DOCA/salt-induced cardiac fibrosis and cardiocyte hypertrophy occur through NHE1 activation. This is the first demonstration of the involvement of NHE1 in the detrimental effects of exposure to DOCA/salt on the heart. Also, from the above data, we conclude that DOCA/salt-induced cardiac fibrosis and hypertrophy are not related to the blood pressure elevation because cariporide, which had no effect on arterial hypertension, did prevent both cardiac fibrosis and hypertrophy. This notion is in good agreement with that of Brilla et al.5

Cardiac NHE represents one of the heart’s key components to maintain physiological pH. Under conditions of myocardial ischemia, this physiological mechanism appears to exert detrimental effects on the myocardium, probably by increasing the intracellular Na+ load, which finally results in elevated intracellular Ca2+ via Na+/Ca2+ exchange.5 Numerous studies with various NHE1 inhibitors demonstrated protective effects of NHE1 inhibition in animal models of myocardial ischemia.5 NHE1 inhibitors have also been demonstrated to exert protective actions in a postinfarction model.5,15 Although the drug was administered during the ischemic period, there was still a beneficial effect on both interstitial and perivascular fibrosis and cardiocyte hypertrophy in nonischemic regions of the myocardium. Thus, one might speculate that part of the protective effect of NHE1 inhibition in this model might have been independent of the drug’s action on ischemic cardiocytes. Indeed, the present work supports the notion that NHE1 is involved in the formation of hypertrophy and fibrosis of nonischemic origin.

Cardiac fibroblasts are known to express NHE1,16 and it has been shown that mitogenic stimuli activate NHE1 in fibroblasts both at the protein and transcription levels.17 Treatment of cardiovascular cells with aldosterone has been shown to increase NHE1 mRNA levels and NHE activity.11,12 Although the level of NHE1 activity is very low under physiological pH,18 the NHE1 protein expression induced by the exposure to DOCA/salt may increase NHE1 activity to a level where it significantly contributes to the development of cardiocyte hypertrophy and fibrosis. NHE1 activation has also been shown for a variety of mitogenic and growth-promoting stimuli,17 thus clarifying how cariporide suppresses cardiac fibrosis and hypertrophy. The latter is corroborated by our finding that the increased expression of NHE1 was completely normalized after treatment with the NHE1 inhibitor cariporide. This occurred in the presence of continued DOCA/salt stimuli and thus the expression of NHE1 might be stimulated by a mechanism further downstream in the signaling cascade.

In the present study, we observed that cotreatment with spironolactone completely inhibited DOCA/salt-induced arterial hypertension, partially reversed the DOCA/salt-induced increase in heart weight, partially inhibited the DOCA/salt-induced increase in interstitial cardiac fibrosis, and completely prevented the DOCA/salt-induced increase in perivascular cardiac fibrosis. We also showed that spironolactone had no effects on DOCA/salt-induced cardiocyte hypertrophy in the subendocardial or subepicardial regions. These findings indicate that DOCA/salt-induced interstitial and perivascular fibrosis is inhibited by a spironolactone-sensitive mechanism(s), that is, presumably by inhibition of MRs, whereas the DOCA/salt-induced cardiocyte hypertrophy does not. The present study also showed that the DOCA/salt-induced NHE1 protein expression was partially inhibited by treatment with spironolactone, consistent with the notion that NHE1 represents a key downstream factor activated by spironolactone-sensitive MRs, whereas the DOCA/salt-induced NHE1 activation partly occurs through MRs. This is the first demonstration of a link between the MRs and NHE1 in the effects of DOCA/salt on cardiac fibrosis. This notion is also supported by the report of Ebata et al.11 in which the exposure of vascular smooth muscle cells to aldosterone for 24 hours caused increased NHE1 mRNA levels and NHE activity, which were inhibited by the treatment with spironolactone.

Spironolactone had no effect on DOCA/salt-induced cardiac hypertrophy, although it completely inhibited DOCA/salt-induced arterial hypertension. Therefore these results are compatible with the idea that DOCA/salt-induced cardiac hypertrophy occurs through spironolactone-insensitive processes. In vitro studies19,20 reported that aldosterone doubled the rate of synthesis of collagen by cultured cardiac fibroblasts, an effect not antagonized by a 3000-fold excess of the MR antagonist spironolactone, which therefore presumably does not operate through MRs. Recently, rapid, nongenomic effects of aldosterone have been demonstrated in several cell types, including cardiovascular cells.11,21 They were not inhibited by actinomycin D (a DNA transcription inhibitor), in addition to their rapid time course. They have generally been held to reflect aldosterone action through a high affinity membrane receptor, distinct from the classic intracellular MR. Postreceptor mechanisms established include the activation of protein kinase C, leading to the activation of NHE in
vascular smooth muscle cells.\textsuperscript{11,12} Ebata et al.\textsuperscript{11} reported that the exposure of vascular smooth muscle cells to aldosterone for 3 hours did not affect NHE1 mRNA levels but caused an increase in NHE activity that was not inhibited by actinomycin D, cycloheximide (a protein synthesis inhibitor), spironolactone, or RU38486 (a glucocorticoid receptor antagonist). Therefore, it is possible that DOCA/salt-induced cardiac hypertrophy through the NHE1 activation may be mediated by nongenomic mechanisms, in addition to genomic mechanisms. This possibility requires further investigation.

**Perspectives**

Cardiac NHE1 contributes to DOCA/salt-induced cardiac fibrosis and hypertrophy, and the NHE1 inhibitor cariporide completely prevents the detrimental effects of DOCA/salt on cardiac fibrosis and hypertrophy. In addition, DOCA/salt-induced cardiac fibrosis through the MRs partly occurs through NHE1 activation. These findings provide novel insights into the pathogenesis of mineralocorticoid/salt-induced cardiac fibrosis. In addition to its application to prevent postischemic damage to the heart,\textsuperscript{5,13} inhibition of NHE1 activity might represent a novel therapeutic strategy in human cardiac fibrosis associated with hyperaldosteronism.

**References**

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Hypertension. 2003;41:493-498; originally published online February 17, 2003;
doi: 10.1161/01.HYP.0000056769.73726.E5
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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World Wide Web at:
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