Calcineurin is critical for sodium-induced neointimal formation in normotensive and hypertensive rats

Ryo Takeda,1 Etsu Suzuki,2 Masao Takahashi,1 Shigeyoshi Oba,1 Hiroaki Nishimatsu,3 Kenjiro Kimura,2 Tetsuo Nagano,4 Ryozo Nagai,1 and Yasunobu Hirata1

1Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo; 2Institute of Medical Science, St. Marianna University School of Medicine, Kawasaki; and 3Department of Medicine, Faculty of Medicine, and 4Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

Submitted 10 January 2008; accepted in final form 25 April 2008


First published May 2, 2008; doi:10.1152/ajpheart.00031.2008.—It is well known that excessive intake of sodium chloride (sodium) is a risk factor for cardiovascular disease because it raises blood pressure. However, sodium loading reportedly promotes cardiovascular disease independently of its effect on blood pressure. To examine the mechanisms by which sodium loading promotes vascular inflammation independently of its effect on blood pressure, we examined the role of calcineurin in sodium-loading-induced vascular inflammation using a wire injury model of the rat femoral artery. Calcineurin mRNA expression in the wire-injured femoral artery was significantly higher in sodium-loaded normotensive rats, such as Wistar-Kyoto (WKY) rats, than that in control WKY rats. Neointimal formation was also significantly enhanced in sodium-loaded WKY rats compared with control WKY rats. Gene transfer of an adenovirus expressing a dominant negative mutant of calcineurin (AdCalAC92Q) significantly suppressed neointimal formation in sodium-loaded WKY rats to a level similar to that observed in control WKY rats. Calcineurin expression and neointimal formation were more significantly enhanced in hypertensive rats, such as spontaneously hypertensive rats (SHRs), than those in control WKY rats. AdCalAC92Q infection significantly suppressed neointimal formation in SHRs to a level similar to that observed in control WKY rats. These results suggest that sodium loading promotes neointimal formation, even in normotensive rats, and that hypertension further stimulates neointimal formation. These results also suggest that calcineurin plays a pivotal role in this process.

signal transduction; vasculature; inflammation; hypertension

It has been well established that an excess intake of sodium chloride (sodium) induces hypertension in some populations. Although sodium seems to induce cardiovascular disease through the elevation of blood pressure, it has been known that sodium can induce cardiovascular disease independent of blood pressure elevation (23). It has been reported that dietary sodium intake is a potent and independent determinant of cardiac hypertrophy in humans (2, 25). It has also been reported that cardiac hypertrophy occurs by sodium loading in Wistar-Kyoto (WKY) rats without an elevation of systemic blood pressure (12, 29, 33). Furthermore, it has been reported that the pulse wave velocity of normotensive subjects on a low-salt diet was lower than that of a control group, matched for age and blood pressure, on a normal salt diet (1). However, it has not been fully examined whether sodium loading has a blood pressure-independent effect on vascular pathophysiology such as atherosclerosis and restenosis after angioplasty. It also remains unclear about the mechanism whereby sodium loading has a blood pressure-independent effect on the vascular system. Sodium loading may increase the cytosolic Ca2+ concentration via activation of the Na+/Ca2+ exchanger (NCX) (3), which may, in turn, activate Ca2+-dependent pathways such as calcineurin and MAPK. Thus, these Ca2+-dependent pathways may trigger sodium-induced inflammation in blood vessels in a normotensive state. Although hypertension is a major risk factor for cardiovascular disease, the precise molecular mechanisms by which hypertension induces vascular inflammation are not fully understood. It is well known that shear stress and stretch increase the concentration of cytosolic Ca2+ (20, 26, 32) and activate several intracellular signaling pathways, such as calcineurin and MAPK, in a variety of cell types (8, 10, 11, 17–19, 22). Since hypertension increases the stretching of the vascular wall, it may also induce vascular inflammation via Ca2+-dependent pathways.

Calcineurin is a serine/threonine phosphatase that is activated in a Ca2+/calmodulin-dependent manner and is involved in the activation of immune response genes in B and T cells (6, 13, 14). It has become clear that calcineurin plays a pivotal role in the development of cardiac hypertrophy by activating transcription factors called nuclear factors of activated T cells (NFAT). Calcineurin dephosphorylates NFAT, which, in turn, promotes nuclear translocation of NFAT. NFAT transcription factors then cooperate with nuclear transcription factors such as GATA-4 and stimulate the transcriptional activation of various genes involved in the development of cardiac hypertrophy (15). In contrast to its influence on the pathophysiology of the heart, the role of calcineurin in the pathophysiology of blood vessels remains largely unknown.

Monocyte chemoattractant protein (MCP)-1 is a peptide that induces the migration of monocytes/macrophages in the vessel wall and is reportedly implicated in the formation of vascular diseases such as atherosclerosis and restenosis after angioplasty (30). It has been reported that MCP-1 plays a pivotal role in the transcriptional activation of various genes involved in the development of cardiac hypertrophy (15). In contrast to its influence on the pathophysiology of the heart, the role of calcineurin in the pathophysiology of blood vessels remains largely unknown.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
neointimal formation by stimulating the infiltration of macrophages into blood vessels (4, 7, 16). We have recently reported that calcineurin stimulates the expression of MCP-1 in vascular myocytes (24). We have also shown that calcineurin is involved in the formation of neointima after mechanical injury to the rat femoral artery by the upregulation of MCP-1, thus stimulating macrophage infiltration (24).

We therefore hypothesized that a high sodium intake induces vascular inflammation via a calcineurin-dependent pathway even if there is no increase in blood pressure. To test this hypothesis, we used a wire injury model of the rat femoral artery and examined the extent of neointimal formation under low and high sodium intake. We also infected the femoral artery with adenoviruses expressing a dominant negative mutant and a constitutively active mutant of calcineurin and examined their effect on the formation of neointima.

MATERIALS AND METHODS

Reagents. ANG II was purchased from the Peptide Institute (Osaka, Japan).

Animals. All animal experiments were performed with the approval of and in accordance with the guidelines for animal care of Tokyo University. Male WKY rats and spontaneously hypertensive rats (SHRs) were purchased from Charles River Laboratories (Wilmington, MA). Each of these strains was divided into two groups: a control group maintained on tap water and a sodium-loaded group given drinking water containing 0.9% sodium chloride. Rats in each group received standard chow containing 0.53% sodium chloride. Rats in the sodium-loaded groups received 0.9% sodium chloride solution beginning at the age of 4 wk and continuing until the animals were 10 wk of age. A wire injury of the rat femoral artery was induced at the age of 8 wk.

Cell culture. Vascular smooth muscle cells (VSMCs) were cultured from rat thoracic aortas following the explant method, as previously described (21).

Construction of a dominant negative mutant of human calcineurin A. Details of the cloning of amino-terminally hemagglutinin epitope-tagged human calcineurin A 1-398 (CaLaΔC), a constitutively active mutant lacking the carboxyl-terminal calmodulin binding domain, have been previously described (28). To construct a dominant negative mutant of human calcineurin A, His92 of CalAΔC was replaced with glutamine (CaLAΔC92Q) (31). The primer used to introduce the point mutation was as follows: 5’-CTGTGGTTGGGAGACCATTCAAGGACATTTGATTG-3’ (where the bold lowercase letter indicates the nucleotide substitution to introduce the point mutation). The entire DNA sequence was determined by a cycle sequence reaction using a CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, CA).

Construction of a replication-defective adenovirus. Construction of a replication-defective adenovirus that expressed a constitutively active mutant of human calcineurin A (AdCaLaΔC) has been described previously (28). A replication-defective adenovirus that expressed CaLaΔC92Q (AdCaLaΔC92Q) was constructed according to the method previously described using an AdMax kit (Microbix Biosystems, Toronto, ON, Canada) (28). A recombinant adenovirus that expressed green fluorescence protein (AdGFP) was obtained from Cell Biolabs (San Diego, CA).

Measurement of calcineurin activity. Calcineurin activity was measured using an assay kit (BioMol, Plymouth Meeting, PA) according to the instructions provided by the manufacturer. In brief, after cultured cells had been in a phosphate-free buffer [20 mmol/l Tris (pH 7.2) and 150 mmol/l NaCl], proteins were extracted from cultured cells with a lysis buffer [50 mmol/l Tris–HCl (pH 7.5), 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l dithiothreitol, and 0.2% Nonidet P-40] provided by the manufacturer. To remove phosphatase completely, protein extracts were applied to a gel filtration chromatography column. Desalted protein extracts were used for the assay. Phosphatase activity was measured using RII phosphopeptide as a substrate in the presence of 0.5 μmol/l okadaic acid (OA) and both 0.5 μmol/l OA and 10 mmol/l EGTA (OA-EGTA). The phosphatase activity in OA-EGTA treatment was subtracted from that in OA treatment, and the subtracted phosphatase activity was regarded as calcineurin activity.

Femoral arteries. Femoral arteries were fixed by perfusion of 4% paraformaldehyde and processed for paraffin embedding. Cross sections (2 μm) were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin. For immunohistochemistry, sections were incubated with mouse anti-rat ED1 antibody (Serotec, Oxford, UK) diluted at 1:800. Sections were then incubated with biotinylated anti-mouse secondary antibody and finally horseradish peroxidase-labeled streptavidin according to the instructions provided by the manufacturer (DAKO, Copenhagen, Denmark). Sections were counterstained with hematoxylin.

RNA extraction and real-time PCR. Total RNA was extracted using TRIzol reagent (GIBCO-BRL, Rockville, MD) according to the instructions provided by the manufacturer. To extract total RNA from the rat femoral artery, the femoral artery was homogenized in TRIzol reagent. After phenol-chloroform extraction, a small amount of total RNA was coprecipitated with tRNA (Sigma, St. Louis, MO). Total RNA was subjected to reverse transcription using an Omniscript RT kit (Qiagen, Tokyo, Japan). The expression of MCP-1 and GAPDH was examined by real-time PCR using SYBR green dye as previously described (24). The PCR primers used to amplify rat calcineurin A were as follows: sense 5’-TGTTGAGGCAGCCCATTT-3’ and antisense 5’-CCCAGTGATTAACACTCTCC-3’.

Western blot analysis. Western blot analysis was performed as previously described (27).

Statistical analysis. Values are means ± SE. Statistical analyses were performed using ANOVA followed by Student-Neumann-Keuls multiple comparison test. Significance was determined using the Student’s t-test.

Fig. 1. Time course of systolic blood pressure. The systolic blood pressure of control [NaCl (−)] and sodium-loaded [NaCl (+)] rats was measured by the tail-cuff method (n = 6 each). *P < 0.05 vs. control Wistar-Kyoto (WKY) rats; #P < 0.05 vs. control spontaneously hypertensive rats (SHRs).
Keul’s test. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

Time course of blood pressure. The systolic blood pressure of WKY rats measured by the tail-cuff method was not significantly altered by sodium loading at any time point examined. The systolic blood pressure of SHRs slightly but significantly increased by sodium loading compared with control SHRs from the ages of 8 to 10 wk (Fig. 1). Body weight and heart rate were not significantly changed by sodium loading in WKY rats or SHRs (data not shown).

Sodium loading promotes neointimal formation in normotensive rats. We next examined the effect of sodium loading on neointimal formation in the rat femoral artery. Neointimal formation (ratio of the intimal area to the medial area) significantly increased in sodium-loaded WKY rats compared with that in control WKY rats (Fig. 2). The extent of neointimal formation observed in sodium-loaded WKY rats was similar to that observed in control SHRs. Neointimal formation in control SHRs significantly increased compared with that in control WKY rats. The extent of neointimal formation in sodium-loaded SHRs was significantly greater than that in sodium-loaded WKY rats. Sodium loading tended to increase neointimal formation in SHRs, although the difference was not statistically significant. These results suggest that sodium loading stimulates neointimal formation even in normotensive rats and that hypertension per se also stimulates neointimal formation.

Calcineurin expression in the femoral artery. We originally tried to measure calcineurin activity in the wire-injured femoral artery. However, calcineurin activity was under detectable levels, since a very little amount of protein was extracted from the femoral artery. We therefore examined calcineurin mRNA expression in the femoral artery by real-time PCR. Calcineurin A expression in the wire-injured femoral artery was significantly increased in sodium-loaded WKY rats, control SHRs, and sodium-loaded SHRs com-
pared with control WKY rats (Fig. 3), suggesting the possibility that sodium loading stimulates calcineurin activity in the wire-injured femoral artery and that hypertension per se also stimulates calcineurin activity.

Sodium loading stimulates neointimal formation via a calcineurin-dependent pathway. Since we found that calcineurin is implicated in neointimal formation (24), we next examined the role of calcineurin in neointimal formation induced by sodium loading. We used an adenovirus construct that expresses a dominant negative mutant of human calcineurin A (AdCalAΔC92Q). The expression of CalAΔC92Q was confirmed by immunoblot analysis (Fig. 4A). We first examined whether AdCalAΔC92Q infection could sufficiently inhibit calcineurin activity in cultured rat VSMCs. AdCalAΔC92Q infection significantly suppressed ANG II-induced activation of calcineurin (Fig. 4B). Infection of an adenovirus that expressed a constitutively active mutant of human calcineurin A (AdCalAΔC) significantly increased calcineurin activity in the absence of ANG II stimulation. We therefore infected the rat femoral artery with AdCalAΔC92Q and examined the effect of this on neointimal formation. AdCalAΔC92Q infection, as opposed to AdGFP infection, significantly inhibited neointimal formation in the femoral artery of control WKY rats (Fig. 5). This result was compatible with our previous finding that calcineurin is implicated in neointimal formation in Wistar rats (24). AdCalAΔC92Q infection also significantly inhibited neointimal formation in sodium-loaded WKY rats and did so to the same level as that observed in control WKY rats. Furthermore, AdCalAΔC92Q infection significantly suppressed neointimal formation in control and sodium-loaded SHRs to a similar level as that observed in control WKY rats.

Sodium loading stimulates macrophage infiltration in the neointima via a calcineurin-dependent pathway. Since we have reported that calcineurin is implicated in MCP-1 expression and macrophage infiltration in blood vessels (24), we examined whether sodium loading increased macrophage infiltration in the neointima and whether blockade of calcineurin inhibited macrophage infiltration. Macrophage infiltration in the neointima significantly increased in sodium-loaded WKY rats compared with that in control WKY rats (Fig. 6). AdCalAΔC92Q infection compared with AdGFP infection also significantly suppressed macrophage infiltration in control and sodium-loaded WKY rats. Macrophage infiltration also significantly increased in control SHRs compared with control WKY rats. AdCalAΔC92Q infection compared with AdGFP infection also significantly suppressed macrophage infiltration in control and sodium-loaded SHRs.

Sodium loading stimulates MCP-1 expression in the femoral artery via a calcineurin-dependent pathway. We also examined whether sodium loading induced MCP-1 expression in the wire-injured femoral artery using real-time PCR analysis. MCP-1 expression significantly increased in sodium-loaded WKY rats compared with that in control WKY rats (Fig. 7). MCP-1 expression also significantly increased in control SHRs compared with that in control WKY rats. AdCalAΔC92Q infection significantly suppressed MCP-1 expression in all groups.
Forced activation of calcineurin stimulates neointimal formation, MCP-1 expression, and macrophage infiltration.

To further examine the role of calcineurin in neointimal formation, we infected femoral arteries of control WKY rats with AdCalAΔC and examined the effect on neointimal formation. AdCalAΔC infection compared with AdGFP infection significantly stimulated neointimal formation in control WKY rats (Fig. 8, A and B). AdCalAΔC infection compared with AdGFP infection also significantly promoted macrophage infiltration in control WKY rats (Fig. 8, C and D). Furthermore, AdCalAΔC infection compared with AdGFP infection significantly promoted MCP-1 expression in control WKY rats (Fig. 8E). These results supported our hypothesis that sodium loading increases neointimal forma-
tion, MCP-1 expression, and macrophage infiltration via calcineurin activation in blood vessels.

**DISCUSSION**

In the present study, we showed that sodium loading promoted neointimal formation in the wire injury model of the rat femoral artery. A high sodium intake significantly stimulated neointimal formation even in WKY rats, whose blood pressure was unaltered by sodium loading. In parallel with these results, calcineurin expression in the femoral artery was significantly increased by sodium loading; in WKY rats, it increased without any change in their blood pressure. Furthermore, blockade of calcineurin activity by AdCalΔC92Q infection significantly inhibited neointimal formation in sodium-loaded WKY rats to a level similar to that in control WKY rats. These results suggest that sodium loading induces neointimal formation independent of its effect on blood pressure and that calcineurin plays a critical role in sodium-induced neointimal formation. It
has been shown that MCP-1 plays a pivotal role in neointimal formation by stimulating the infiltration of macrophages into blood vessels (7, 16). We therefore examined macrophage infiltration in the neointima and MCP-1 expression in the wire-injured femoral artery. Macrophage infiltration significantly increased in the neointima of sodium-loaded WKY rats compared with control WKY rats, and AdCalΔC92Q infection significantly suppressed macrophage infiltration. In accordance with these results, MCP-1 expression in the femoral artery significantly increased in sodium-loaded WKY rats compared with control WKY rats, and AdCalΔC92Q infection significantly suppressed MCP-1 expression. Furthermore, AdCalΔC infection significantly promoted neointimal formation, MCP-1 expression, and macrophage infiltration in normotensive control WKY rats. Thus, our results indicate that sodium loading promotes neointimal formation independently of its effect on blood pressure, at least partly by activating calcineurin and the resultant MCP-1 expression and macrophage infiltration.

Calcineurin expression, neointimal formation, macrophage infiltration, and MCP-1 expression were significantly enhanced in control SHRs compared with control WKY rats. AdCalΔC92Q infection significantly and effectively suppressed neointimal formation, MCP-1 expression, and macrophage infiltration in control and sodium-loaded SHRs. These results suggest that hypertension per se stimulates calcineurin expression, neointimal formation, MCP-1 expression, and macrophage infiltration and that calcineurin is also critically implicated in neointimal formation in hypertensive rats as well as in normotensive rats through the stimulation of MCP-1 expression and macrophage infiltration. Unexpectedly, sodium loading did not significantly stimulate neointimal formation, MCP-1 expression, or macrophage infiltration in SHRs, probably because the intracellular signaling pathways that mediate neointimal formation, such as calcineurin, were sufficiently activated in control SHRs to induce a maximal level of neointimal formation.

The precise mechanism by which sodium loading stimulates neointimal formation remains unclear. Our results suggest that sodium loading promotes neointimal formation through stimulating the calcineurin-dependent pathway, at least partly, via upregulation of calcineurin expression. Sodium loading may also stimulate other intracellular signaling pathways, such as MAPK, that mediate neointimal formation. Activation of these pathways, together with calcineurin, may further stimulate neointimal formation. However, the activation of calcineurin is still necessary, because AdCalΔC92Q infection potently inhibited neointimal formation in sodium-loaded WKY rats and SHRs. Activation of other signaling pathways alone by sodium loading, even if it happens, does not seem to be sufficient to induce neointimal formation. Thus, the activation of calcineurin seems to be critical for sodium-induced neointimal formation. Sodium loading may also stimulate calcineurin activity via an increase in cytosolic Ca\(^{2+}\) concentration. There are several possible explanations. First, a high sodium intake increases cytosolic Ca\(^{2+}\) concentration via the activation of NCX (3), which may result in the activation of calcineurin. It has recently been shown that type 1 NCX seems to be implicated in Ca\(^{2+}\) mobilization into the cytosolic space from extracellular fluid in sodium-dependent hypertension (9). Second, increased blood pressure (pressure overload) augments stretch to the vessel wall, which may activate calcineurin (8, 10, 11, 17–20, 22, 26, 32). Along these lines, it has been reported that pressure overload activates calcineurin in the heart. Pressure overload induced by aortic banding reportedly stimulated cardiac calcineurin activity and cardiac hypertrophy, whereas blockade of calcineurin activation ameliorated cardiac hypertension (5, 34). Future studies will be needed to examine these possibilities.

Because the wire injury model is not a model of atherosclerosis, our results do not directly indicate the role of calcineurin in sodium-induced progression of atherosclerosis. Studies using a model of atherosclerosis such as apolipoprotein E-null mice will be required to clarify this point.

Although the restriction of dietary sodium intake has been recommended to hypertensive patients, especially those who are sodium sensitive, it remains to be determined whether sodium intake should be restricted in the normotensive population. Our results suggest that the intake of excessive amounts of sodium can induce cardiovascular diseases even in the normotensive population. Thus, it appears that sodium intake should be restricted in normotensive patients, especially those who have other cardiovascular risk factors, such as diabetes.

In conclusion, sodium loading induces neointimal formation even in normotensive state via a calcineurin-dependent pathway, and hypertension further promotes neointimal formation. Although several calcineurin inhibitors are clinically used as immunosuppressants, they have some adverse effects, such as nephrotoxicity and hypertension. It will be necessary to develop novel calcineurin inhibitors to reduce cardiovascular events.

**REFERENCES**


