Calcineurin Blockade Prevents Cardiac Mitogen-activated Protein Kinase Activation and Hypertrophy in Renovascular Hypertension*

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Chronic stimulation of the renin-angiotensin system induces an elevation of blood pressure and the development of cardiac hypertrophy via the actions of its effector, angiotensin II. In cardiomyocytes, mitogen-activated protein kinases as well as protein kinase C isoforms have been shown to be important in the transduction of trophic signals. The Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin has also been suggested to play a role in cardiac growth. In the present report, we investigate possible cross-talks between calcineurin, protein kinase C, and mitogen-activated protein kinase pathways in controlling angiotensin II-induced hypertrophy. Angiotensin II-stimulated cardiomyocytes and mice with angiotensin II-dependent renovascular hypertension were treated with the calcineurin inhibitor cyclosporin A. Calcineurin, protein kinase C, and mitogen-activated protein kinase activations were determined. We show that cyclosporin A blocks angiotensin II-induced mitogen-activated protein kinase activation in cultured primary cardiomyocytes and in the heart of hypertensive mice. Cyclosporin A also inhibits specific protein kinase C isoforms. In vivo, cyclosporin A prevents the development of cardiac hypertrophy, and this effect appears to be independent of hemodynamic changes. These data suggest cross-talks between the calcineurin pathway, the protein kinase C, and the mitogen-activated protein kinase signaling cascades in transducing angiotensin II-mediated stimuli in cardiomyocytes and could provide the basis for an integrated model of cardiac hypertrophy.

Chronic elevated blood pressure results in structural changes of the cardiovascular system. In particular, cardiac hypertrophy is thought to represent an adaptation to increased workload. However, in the longer term, this process might evolve into heart failure with increased risk of mortality. The renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis through the renin-angu...
In the present study, we used mouse primary cardiomyocytes and a murine model of renovascular hypertension (22) to investigate whether CaN could play a role in cardiac hypertrophy. Results demonstrate that CsA inhibits Ang II-induced MAPK activation in primary cardiomyocytes as well as in cardiac tissue possibly via a PKC-dependent mechanism. In addition, CaN blockade also prevents the development of cardiac hypertrophy during renovascular hypertension. Taken together, these results suggest cross-talks between the CaN and the MAPK pathways for Ang II-stimulated cardiac hypertrophy.

**MATERIALS AND METHODS**

**Mice and CsA Treatment**—Eight-week-old, male C57BL/6 mice (Iffa Credo, L’Arbresle, France) were injected intraperitoneally with either saline or 25 mg of CsA (Sandimmun®, Novartis, Switzerland/kg of body weight twice daily starting on the day of clipping. Mice were maintained on sterile tap water and regular rodent diet *ad libitum*.

**Cell Culture**—Neonatal C57BL/6 mouse ventricles were separated from atria. Cardiomyocytes and nonmyocyte cells were purified as described (23) and plated at a density of 0.1 × 10^6 cells/cm² on gelatin-coated wells and noncoated wells, respectively. After 24 h in culture, cells were switched to no serum medium, and Ang II was added 24 h later. Nonmyocyte cells were passaged once before the addition of no serum medium. Cells were pretreated with either 250 ng/ml CsA or 150 ng/ml FK506 for 20 min prior to the addition of 100 nM Ang II. Stimulation time was 5 min for p38 and ERK and 10 min for JNK. Cells were then washed with phosphate-buffered saline and lysed in radioimmuno precipitation buffer (150 mM NaCl, 0.25% deoxycholic acid, 1% Triton X-40, 1 mM NaVO₃, 1 mM NaF, 1 µg/ml aprotinin, 1 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 mM EDTA, 50 mM Tris, pH 7.5). The lysates were either heated at 95 °C for 5 min for SDS-PAGE or kept frozen until used. In some experiments, cells were cultured in absence of calcium. In this case, cardiomyocytes were switched to calcium-free medium and then treated for 2 h, with 100 µM thapsigargin.

**Surgery**—Briefly, mice were anesthetized using 1.5% halothane in oxygen. The left kidney was exposed, and a clip (0.12-mm opening) was inserted on the renal artery isolated by blunt dissection as described (22). Sham procedure included the entire surgery with the exception of artery clipping. For blood pressure and heart rate measurement, the right carotid artery was exposed through cervical incision. A silicon catheter filled with 5% glucose solution containing heparin (300 IU/ml) was inserted into the vessel and tunneled subcutaneously to exit at the back of the neck. Blood pressure and heart rate were recorded in conscious mice by connecting the catheter to a pressure transducer using a computerized data acquisition system (Notocord, Paris, France).

**Tissue Sampling**—Heart and kidneys were frozen in liquid nitrogen and stored at −70 °C until used. Heart wet weight (without the atria) was normalized to the tibial length and to body weight.

**Cardiac Protein Extract**—Hearts were rapidly excised and rinsed in cold phosphate-buffered saline. Atria were removed, and ventricles were homogenized on ice in 1 ml of radioimmune precipitation buffer. Homogenates were sonicated on ice for three bursts of 5 s each and centrifuged for 15 min at 4200 rpm at 4 °C. Lysates were kept frozen until used. The activity of the different PKC isoforms were detected using a computerized data acquisition system (Notocord, Paris, France).

**MAPK Activation**—Protein concentrations were determined according to Bradford. Thirty µg of soluble proteins were run in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Biorad, nitrocellulose; Amersham Pharmacia Biotech). Phosphorylated MAPK were detected by Western blot analysis using specific antibodies for the phosphorylated forms of p38, ERK, and JNK (New England Biolabs). After overnight incubation at 4 °C in blotto (20 mM Tris, pH 7.6, containing 0.1% Tween 20 and 5% dry milk), first antibodies were revealed using anti-rabbit secondary antibodies conjugated to horseradish peroxidase and a chemiluminescent detection system (Amersham Pharmacia Biotech). MAPK phosphorylation was quantified by laser-scanning densitometry of specific phosphorylated bands (NIH Image).

**PKC Activation**—Cells were lysed in 50 µl in 12.5 mM Tris, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM dithiothreitol, 300 µM phenylmethylsulfonyl fluoride, 120 µM pepstatin A, 200 µg/ml leupeptin, 200 µg/ml aprotinin, pH 7.4. The extracts were incubated for 5 min at 4 °C and centrifuged at 12,000 rpm for 15 min at 4 °C. Soluble fractions were heated at 95 °C for 5 min in 2× SDS loading buffer. Particular fractions were washed once in lysis buffer, resuspended in 50 µl of lysis buffer containing 1% Triton X-100, and heated at 95 °C for 5 min in 2× SDS loading buffer. Samples were run in SDS-PAGE and transferred on nitrocellulose membrane as above. PKC isoforms were detected using isoform-specific antibodies (Biomol). The activity of the different PKC isoforms was determined using a computerized data acquisition system (Notocord, Paris, France). The activity of the different PKC isoforms was determined using a computerized data acquisition system (Notocord, Paris, France).
isoforms (α, β, ε, ζ) in vivo was measured by immunoprecipitating from cardiac protein extracts each isoform using specific antibodies (Biomol). Activity was assayed at 30 °C in the presence of 50 μM ATP, 10 μCi of [γ-32P]ATP, and myelin basic protein (Upstate Biotechnology, Inc., Lake Placid, NY) (24). The amounts of 32P-labeled peptide were measured by liquid scintillation counting. To block the different PKC isoforms, cardiomyocytes were treated with 2 μM of isoform-specific PKC inhibitors (Biomol) for 24 h.

Calcineurin Assay—Assay was performed essentially as described (21). Briefly, hearts or cultured cardiomyocytes were homogenized in 1 ml of assay buffer (0.1 M MOPS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, containing a protease inhibitor mixture (Complete; Roche Molecular Biochemicals)). Soluble cardiac extracts were incubated for 20 min at 30 °C in 20 mM Tris, 100 mM KCl, 6 mM MgCl2, 100 μM CaCl2, 500 μM dithiothreitol, 100 nM calmodulin, 500 nM okadaic acid, pH 8, in the presence of 2×106 cpm of [γ-32P]ATP-labeled RII peptide. Free 32P was separated from RII substrate using a Dowex AG 50W-X8 cation exchange resin (Bio-Rad). Remaining phosphorylated RII substrate was quantitated by scintillation counting.

Northern Blot—Total RNA was purified with TriPure (Roche Molecular Biochemicals). Ten μg of total RNA was separated on 1% formamide-agarose gel and transferred to GeneScreen nylon membranes (PerkinElmer Life Sciences). Kidney RNAs were hybridized with a radiolabeled mouse renin probe (kindly provided by Dr. K. Nakayama, University of Tsukuba, Japan), and heart RNAs were hybridized with a mouse brain natriuretic peptide (BNP) probe. The intensity of hybridization signals was quantified with an Instant Imager detector (Packard Instruments). The blots were then stripped and hybridized with a mouse glyceraldehyde-3-phosphate dehydrogenase probe for normalization.

Statistical Analysis—All values are expressed as means ± S.E.
Group means were compared by analysis of variance using a Student-Newman-Keuls test for post hoc comparison. \( p \) values, \( 0.05 \) were considered significant.

RESULTS

Effect of CsA on CaN and MAPK Activation in Ang II-stimulated Primary Cardiac Myocytes and Nonmyocyte Cells—To investigate possible cross-talks between the Ang II-activated MAPK and CaN pathways, cardiac myocytes and nonmyocyte cells were stimulated with Ang II, and phosphorylation of p38, ERK, and JNK was measured. All three kinases were activated in cardiac myocytes in response to Ang II (Fig. 1, A–D). In contrast, in nonmyocyte cells, p38 was not activated (Fig. 2, A–D). In addition, Ang II stimulation led to a marked increase in CaN activity in myocytes (Table I). This activity was completely inhibited in the presence of CsA. Blockade of the CaN pathway in cardiomyocytes using CsA resulted in the complete inhibition of the activation of all three MAPK by Ang II (Fig. 1, A–D). To exclude nonspecific inhibition of CsA on MAPK activation, we studied the effects of FK506, a different CaN blocker (14). This drug was as effective as CsA in inhibiting Ang II-induced MAPK activation in cardiomyocytes (Fig. 1, A–D). Similarly, ERK and JNK phosphorylation was inhibited by CsA treatment in nonmyocyte cells (Fig. 2, A–D).

Effect of CsA on PKC Activation in Ang II-stimulated Primary Cardiomyocytes—To investigate whether PKC could be affected by CsA treatment, we determined the activity of two

FIG. 4. Inhibition of MAPK activation in Ang II-stimulated (100 nM) cardiomyocytes treated with isoform-specific PKC inhibitors (\( \pi \)inh, \( \epsilon \)inh, and \( \zeta \)inh). Results are expressed as percentage of phosphorylation ± S.E. *, \( p < 0.05 \) as compared with unstimulated controls (A–C). D, Western blot analysis of MAPK activation; the phosphorylated and total p38, ERK, and JNK proteins were detected using specific antibodies.

FIG. 5. CsA prevents renovascular hypertension-induced cardiac hypertrophy. Mean blood pressure (A), heart weight to tibial length ratio (B), and heart weight to body weight ratio (C) in sham and 2K1C mice were determined 4 weeks after clamping in mice injected with either saline or 25 mg of CsA/kg of body weight twice daily. Results are expressed as mean ± S.E. \( n = 7–14 \). *, \( p < 0.05 \) as compared with sham-operated groups.
Calcineurin in Cardiac Hypertrophy

Effect of CsA treatment on cardiac hypertrophy and activation of MAPKs in vivo

| Days after clipping | Heart weight (g) | Tibial length (mm) | HW/TL* ratio |
|---------------------|-----------------|-------------------|--------------|
| Sham untreated      | 9.1 ± 1.3       | 17.5 ± 0.3        | 0.63 ± 0.05  |
| Clipped untreated   | 10.2 ± 0.4      | 17.7 ± 0.2        | 0.61 ± 0.04  |
| Sham CsA-treated    | 9.0 ± 0.5       | 17.4 ± 0.1        | 0.61 ± 0.03  |
| Clipped CsA-treated | 10.2 ± 0.2      | 17.4 ± 0.2        | 0.62 ± 0.05  |

MAPK activities are expressed as means of fold-increase values ± S.E.; *, p < 0.05 compared with sham untreated group.

Calcineurin in Cardiac Hypertrophy

Calcineurin (CaN) is a calcium-dependent protein phosphatase that plays a role in the development of cardiac hypertrophy. CsA, a calcineurin inhibitor, was administered to 2K1C mice to study the effect on cardiac hypertrophy and MAPK activation.

**Effect of CsA on CaN, PKC Isoform, and MAPK Activation in Primary Cardiomyocytes**

To determine whether the PKC isoforms, which were inhibited by CsA, were implicated in Ang II-induced MAPK activation in cardiac hypertrophy, we used a series of isoform-specific PKC inhibitors. Blockade of the activity of calcium-independent PKC isoforms (PKCα and -β) completely abolished Ang II-stimulated MAPK activation (Fig. 4, A–D). In contrast, the inhibition of a calcium-dependent PKC isoform (PKCδ) had no effect on MAPK stimulation by Ang II (Fig. 4, A–D).

**Hypertension and Cardiac Hypertrophy in CsA-treated 2K1C Mice**

To study the possible role of CaN in the development of cardiac hypertrophy induced by a chronic elevation of blood pressure, CsA was administered to sham-operated and 2K1C mice with Ang II-dependent hypertension. Four weeks after clipping, a significant increase in systemic blood pressure was observed in untreated 2K1C mice (Fig. 5A). In these animals, elevated pressure resulted in the development of cardiac hypertrophy (Fig. 5B and C, and Table II). In contrast, CsA treatment completely blocked the increase in cardiac mass. To exclude the possibility that a detrimental effect of CsA on animal growth could represent a confounding factor in these experiments, the development of cardiac hypertrophy was followed by calculating both heart weight to tibial length as well as heart weight to body weight ratios. Both parameters showed consistent CsA-dependent inhibition of cardiac growth. In addition, body weights did not significantly vary in CsA-treated animals (body weight in sham untreated: 25.4 ± 0.7 g; sham CsA-treated: 24.7 ± 0.3 g; clipped untreated: 25.2 ± 0.7 g; clipped CsA-treated: 24.3 ± 0.7 g). Importantly, blood pressure was as high in 2K1C mice receiving CsA as that measured in untreated 2K1C mice (Fig. 5A). To further evaluate the effect of CsA on cardiac tissues, BNP expression was measured as an index of cardiac hypertrophy (Fig. 6A). Clipped animals showed a 3-fold increase in BNP expression in cardiac tissues as compared with controls. In contrast, CsA-treatment blocked stimulated BNP transcription in 2K1C mice. To verify if the type of hypertension that developed in CsA-treated 2K1C mice was still renin-dependent, renin expression in the clipped kidney was determined. Renin mRNA levels were significantly elevated in both untreated and CsA-treated 2K1C mice as compared with values observed in respective control animals (Fig. 6B).

**Effect of CsA on CaN, PKC Isoform, and MAPK Activation in the Heart of 2K1C Mice**

We first confirmed that CsA treatment resulted in CaN inhibition in vivo. Data in Table I demonstrated that CaN activity in the heart of 2K1C mice was indeed completely inhibited by CsA administration. MAPK activation was then followed during the development of cardiac hypertrophy in these mice. We first confirmed that p38, ERK, and JNK were activated in response to chronic hypertension.
(Table II and Fig. 7). Significant activation of both ERK and JNK was observed 3 days after clipping. In contrast, p38 appeared significantly activated only after 4 weeks. MAPK phosphorylation correlated with the progression of cardiac hypertrophy. Interestingly, maximum cardiac enlargement occurred concomitantly with p38 activation. Since CsA treatment prevented the increase in cardiac mass induced by renovascular hypertension (Fig. 5 and Table II) and inhibited MAPK phosphorylation in vitro (Figs. 1 and 2), we studied whether CaN blockade interfered with MAPK activation in the heart of 2K1C mice. Stimulation of all three MAPK pathways was fully inhibited by CsA treatment (Table III). In contrast, calcium-dependent PKC isoforms (PKCα and -β) were not affected. On the contrary, in accordance to what observed in vitro, these isoforms were significantly activated by CsA (Fig. 3 and Table III).

DISCUSSION

Chronic elevation of blood pressure induces the development of cardiac hypertrophy, which appears to depend on MAPK activation. In the present study, we show that CaN blockade inhibits MAPK activation and prevents the development of cardiac hypertrophy in renovascular hypertension, indicating points of convergence between these two pathways. The fact that a different calcineurin inhibitor, namely FK506, also blocks MAPK activation in cardiomyocytes argues against a nonspecific inhibitory effect of CsA (Fig. 1). Ang II-induced MAPK activation appears to depend on the stimulation of calcium-independent PKC isoforms (Fig. 4 and Table III). Since the activity of these particular PKC isoforms was found to be blocked by CsA, it suggests that CsA-mediated inhibition of MAPK activation could result from an inhibitory effect of CsA on calcium-independent PKC isoforms.

Since the initial observation on the preventive effect of CsA on the development of cardiac hypertrophy in transgenic mice (10), results from studies using more physiological models have been reported. Some studies fail to demonstrate an inhibitory effect of CsA on hypertrophy (15, 17, 19). The effectiveness of the CsA treatment could depend on the duration of the hypertrophic stimulus. In mice with transverse aortic constriction, pressure overload-induced cardiac hypertrophy was also insensitive to CsA treatment (18, 20, 21). However, constriction of the aortic arch does not activate the renin-angiotensin system (25). In contrast, a recent study demonstrated a role for CaN in a model of cardiac hypertrophy following abdominal banding known to be partially Ang II-dependent (16). Accordingly, our study describes the inhibitory effect of CsA in an Ang II-dependent model (22). Therefore, it is possible that CaN inhibition effectively blocks Ang II-mediated cardiac hypertrophy, such as that developing in response to abdominal aortic banding or following reduced renal perfusion in renovascular hypertension, and is less effective on mechanical stretch-stimulated cardiac growth. It is noteworthy that CsA treatment does not affect renin expression, since renin mRNA in the kidney of CsA-treated 2K1C animals is as high as that observed in untreated 2K1C mice (Fig. 6B). This suggests that CsA prevention of Ang II-induced cardiac hypertrophy does not result from an inhibition of the activity of the renin-angiotensin system. Although CsA has been reported to increase renin secretion and synthesis, our result is consistent with other studies in CsA-treated rats, in which intrarenal renin mRNA and plasma renin activity were elevated for 2 weeks but not different from that of controls by 4 weeks (reviewed in Ref. 26).

Since CsA was shown to induce anorexia (27), we were concerned about possible toxic effects that could result in nonspecific reduction of cardiac tissues. We think that this is unlikely to play a significant role in the present experiments for several reasons. First, heart weight normalized to tibial length or body weight gave concordant results (Fig. 5, B and C). Second, CsA

| Group                  | PKCα       | PKCβ       | PKCe      | PKCζ      |
|------------------------|------------|------------|-----------|-----------|
| Sham untreated         | 1.00 ± 0.07| 1.09 ± 0.09| 1.00 ± 0.12| 1.00 ± 0.05|
| Clipped untreated      | 1.41 ± 0.10*| 1.57 ± 0.04*| 1.69 ± 0.07*| 1.42 ± 0.06*|
| Sham CsA treated       | 1.31 ± 0.07*| 1.36 ± 0.05*| 1.07 ± 0.04| 1.09 ± 0.12|
| Clipped CsA treated    | 1.47 ± 0.09*| 1.55 ± 0.07*| 1.01 ± 0.04| 1.06 ± 0.07|

FIG. 6. Cardiac BNP and kidney renin expression in CsA-treated 2K1C mice. Cardiac BNP expression (A) and renin expression (B) in 2K1C mice were determined by Northern blot analysis 4 weeks after clipping. Results are expressed as -fold increase over sham ± S.E. n = 7–14. *, p < 0.05 compared with untreated group.

FIG. 7. Western blot analysis of MAPK activation in the heart of CsA-treated 2K1C mice. Heart proteins were analyzed 4 weeks after clipping. The phosphorylated and total p38, ERK, and JNK proteins were detected using specific antibodies.
Calcineurin in Cardiac Hypertrophy

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