Cardiac Natriuretic Peptides Inhibit Cyclosporine-Induced Production of Endothelin in Cultured Rat Mesangial Cells

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Cyclosporine A (CSA) stimulates vascular endothelial cell production of endothelin-1 (ET-1). The present study was designed to test two hypotheses: (1) CSA stimulates ET-1 secretion in cultured rat mesangial cells, and (2) cardiac natriuretic peptides, atrial and brain natriuretic peptides (ANP and BNP), inhibit the above-mentioned secretion in these cells. CSA stimulated ET-1 secretion in a concentration-dependent manner between 10 and 100 ng/mL. In contrast, high concentrations of CSA (10 and 100 μg/mL) were cytotoxic and failed to stimulate ET-1 secretion. Rat ANP (1-28) and rat BNP-45 exhibited clearly concentration-related inhibition of CSA-induced ET-1 secretion. This inhibition by ANP and BNP was paralled by an increase in the cellular level of cyclic guanosine-3′, 5′-monophosphate (cGMP). Rat ANP (5-25) was less effective than rat ANP (1-28) with respect to inhibiting ET-1 secretion and increasing cellular cGMP. Addition of a cGMP analog, 8-bromo-cGMP, reduced CSA-induced ET-1 secretion. On the other hand, addition of a cyclic adenosine-3′,5′-monophosphate (cAMP) analog, 8-bromo-cAMP, did not affect CSA-induced ET-1 secretion. These findings suggest that CSA in low concentrations stimulates ET-1 production in cultured rat mesangial cells, and that cardiac natriuretic peptides inhibit this stimulated production, probably through a cGMP-dependent process.

ENDOTHELIN-1 (ET-1) is a 21–amino acid vasoconstrictive peptide that was first isolated from porcine vascular endothelial cells.[1] This peptide acts on glomerular mesangial cells[2,3] and vascular smooth muscle cells.[1,4] In cultured mesangial cells, ET-1 binds to its specific receptors, enhances phosphoinositide turnover, and induces contraction and proliferation of these cells.[3] Furthermore, previous studies have demonstrated that ET-1 is produced by cultured rat mesangial cells.[5-7]

Cyclosporine A (CSA), an immunosuppressive agent used widely in organ transplantation, has hemodynamic properties similar to those of ET-1.[8] Upon infusion of CSA into animals, a decrease in glomerular filtration rate and renal blood flow occurs along with an increase in mean arterial blood pressure.[9] Recent evidence indicates that CSA stimulates ET-1 production both in vivo and in vitro and increases urinary excretion of ET-1.[10-14] In addition, CSA-induced functional changes such as glomerular hyperfusion/hypofiltration or cellular proliferation are abrogated with ET-1 antibody and ET receptor antagonist,[10,12,14-16] although the acute hemodynamic response to CSA in anesthetized rats was not affected by ET receptor antagonist.[17] These observations suggest that ET-1 plays a central role in CSA-related glomerular dysfunction.

Atrial natriuretic peptide (ANP) was originally isolated from mammalian hearts.[18,19] A second type of natriuretic peptide was then identified in the porcine brain,[20] brain natriuretic peptide (BNP). It has subsequently been isolated from porcine,[21] rat,[22] and human[23] hearts. ANP and BNP are both secreted through the coronary sinus from the heart,[24,25] but ANP is secreted mainly from the atria and BNP from the cardiac ventricles.[26,27] Soijonmaa et al[28] and we[29] have shown that these cardiac natriuretic peptides inhibit basal or stimulated ET-1 production in cultured vascular endothelial cells. Furthermore, several studies have demonstrated that ANP is beneficial in attenuating side effects of CSA.[30-32]

Accordingly, the current study was designed to test two hypotheses: (1) CSA stimulates ET-1 secretion in cultured rat mesangial cells, and (2) cardiac natriuretic peptides ANP and BNP inhibit the above-mentioned secretion in these cells.

MATERIALS AND METHODS

Materials

Angiotensin II (ANG II), 8-bromo–cyclic guanosine-3′,5′-monophosphate (cGMP), 8-bromo–cyclic adenosine-3′,5′-monophosphate (cAMP), and 3-isobutyl-methylxanthine (IBMX) were purchased from Sigma Chemical (St Louis, MO). RPMI 1640 medium, trypsin, Versene, and fetal calf serum were purchased from GIBCO Laboratories (Grand Island, NY). Flasks were purchased from Becton Dickinson (Oxnard, CA). ET-1, ET-2, ET-3, big ET-1 (porcine, 1-39), rat ANP (1-28), and rat BNP-45 were purchased from Peptide Institute (Osaka, Japan). ET-1 antiserum and rat ANP (5-25) were purchased from Peninsula Laboratories (Belmont, CA). ^{125}I–ET-1 was purchased from Amersham Japan (Tokyo, Japan). The cGMP assay kit was purchased from Yamasa Shoyu (Chiba, Japan).

Mesangial Cell Culture

Glomeruli were isolated from Sprague-Dawley rats that weighed 50 to 100 g by sieving with stainless steel and nylon meshes under sterile conditions, as previously reported.[7] Isolated glomeruli were then cultured in RPMI 1640 medium that contained 20% fetal calf serum and antibiotics. Identity of mesangial cells was confirmed by the following criteria[33]: (1) morphology; (2) typical microfilaments seen by transmission electron microscopy; (3) survival in a medium that contained D-valin substituted for L-valin, which indicated the existence of D–amino acid oxidase; (4) resistance to puromycin

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aminonucleoside (10 μg/mL) but susceptibility to mitomycin (10 μg/mL); (5) presence of receptors specific for ANG II and contraction in response to ANG II; and (6) absence of immunofluorescence with factor VIII antibody. Cultures were maintained at 37°C with atmospheric air and 5% CO_2, and subcultures were performed after treatment with Versene followed by trypsin. Cells formed after three to seven passages were used for the experiment.

Pharmacologic Treatment

The culture medium was removed and cell monolayers were washed twice with serum-free RPMI 1640 medium. Various concentrations of CSA (10, 50, and 100 ng/mL) were added to individual wells and cells were incubated at 37°C for 24 and 48 hours. These concentrations of CSA have been shown to stimulate production of ET-1 in cultured human endothelial cells.[11] In separate experiments, CSA in high concentrations (10 and 100 μg/mL) was also added to individual wells and cells were incubated at 37°C for 24 and 48 hours. In final experiments, various concentrations (10^{-6}, 10^{-8}, and 10^{-10} mol/L) of rat ANP (1-28), rat ANP(5-25), or rat BNP-45 were added to the medium in addition to 50 ng/mL CSA and cells were incubated at 37°C for 24 hours.

All experiments were performed with 2 mL RPMI 1640 medium under quiescent (0.5% fetal calf serum) conditions. After incubation, the medium was aspirated and centrifuged at 3,000 × *g* for 10 minutes, and the supernatant was collected and stored at −80°C until radioimmunoassay.

Measurement of ET-1 Concentration

Immunoreactive ET-1 was extracted as previously described.[34,35] Briefly, 1.5 mL of each sample was diluted with 4 mL 4% acetic acid. After centrifugation, the solution was pumped at the rate of 1 mL/min through a Sep-Pak C_{18} cartridge (Millipore, Milford, MA). After evaporation of the eluate with 86% ethanol in 4% acetic acid by a centrifugal evaporator (Model RD-31, Yamato Scientific, Tokyo, Japan), the dry residue was dissolved in the assay buffer described later. Recovery rate was determined by adding three different quantities of cold ET-1 (10, 50, and 100 pg/mL) to serum-free RPMI 1640 medium. Recovery was 69% ± 2%. ET-1 concentration was determined using ET-1 antiserum and ^{125}I–ET-1 as a tracer. This antibody reacts 100% with ET-1 and cross-reacts 7% with ET-2, 7% with ET-3, and 35% with big ET-1 (porcine, 1-39). The antiserum did not cross-react with somatostatin, β-endorphin, human secretin, ANG II, or CSA.

Radioimmunoassay was performed in an assay buffer of 0.01 mol/L sodium phosphate, pH 7.4, that contained 0.05 mol/L NaCl, 0.1% bovine serum albumin, 0.1% Nonidet P-40, and 0.01% NaN_3, as previously described.[34,35] In brief, rehydrated antiserum (100 μL) was added to 100 μL of the sample or 100 μL of standard ET-1 dissolved in the assay buffer, and the mixture was incubated for 24 hours at 4°C. Approximately 15,000 cpm ^{125}I–ET-1 was added to each reaction and incubated for an additional 24 hours. After this incubation, 100 μL diluted normal rabbit serum and 100 μL diluted goat anti–rabbit IgG were added and the mixture was again incubated for 24 hours. After the third incubation, the precipitate was collected by centrifugation at 1,700 × *g* for 30 minutes. The supernatant was removed by aspiration, and the pellet was counted for ^{125}I with a gamma counter. The detection limit of this assay was 0.2 pg/mL, with a range of 0.2 to 200 pg/mL. Interassay variation was 13%, and intraassay variation was 7%.

ANP, BNP, or CSA did not interfere with the radioimmunoassay.

cGMP Measurement

After preincubation, cell monolayers were washed twice with serum-free RPMI 1640 medium and then stimulated for 30 minutes with different concentrations of ANP or BNP dissolved in RPMI 1640 medium that contained 0.5 mmol/L IBMX. Rapid aspiration and the addition of 2 mL ice-cold 65% methanol stopped the reaction. cGMP levels were determined by radioimmunoassay with the cGMP assay kit, as previously reported.[29]

Calculations and Statistical Analysis

The statistical significance of differences in the results was evaluated by ANOVA, and *P* values were calculated by Scheffe's method.[36] Values are expressed as the mean ± SD.

RESULTS

Effect of CSA on ET-1 Secretion

Confluent cultured mesangial cells secreted ET-1 into the medium in a time-dependent manner (Fig 1). CSA stimulated ET-1 secretion in a concentration-dependent manner between 10 and 100 ng/mL (Fig 1). These concentrations of CSA did not cause detachment of cultured cells. In contrast, CSA in high concentrations (10 and 100 μg/mL) did cause detachment of cells and failed to stimulate ET-1 secretion (Table 1).

Effect of ANP and BNP on ET-1 Secretion and Cellular cGMP After Stimulation With CSA

The effects of rat ANP(1-28), rat ANP(5-25), and rat BNP-45 on ET-1 secretion in cultured mesangial cells treated with CSA 50 ng/mL are shown in Fig 2A. Rat ANP(1-28) and rat BNP-45 inhibited ET-1 secretion in a concentration-dependent manner (ANP: CSA alone, 9.8 ± 1.1 pg/mL; CSA + 10^{-10} mol/L ANP, 9.4 ± 1.0; CSA + 10^{-8} mol/L ANP, 8.0 ± 0.7; CSA + 10^{-6} mol/L ANP, 6.4 ± 0.4; BNP: CSA alone, 9.7 ± 1.0 pg/mL; CSA + 10^{-10} mol/L BNP, 9.3 ± 0.8; CSA + 10^{-8} mol/L BNP, 7.8 ± 0.5; CSA + 10^{-6} mol/L BNP, 6.1 ± 0.5). In cells stimulated with CSA, in parallel to the inhibition of ET-1 secretion, cellular cGMP increased after treatment with rat ANP(1-28) or rat BNP-45 (Fig 2B). Rat ANP(5-25) was less effective than the other peptides at inhibiting ET-1 secretion (Fig 2A) and increasing cGMP (Fig 2B).

Effects of a cGMP Analog and a cAMP Analog on CSA-Induced ET-1 Secretion

To determine whether inhibitory effects of ANP and BNP on CSA-induced ET-1 secretion are causally linked to the increase in cellular cGMP, we examined the effect of a cGMP analog, 8-bromo-cGMP, on ET-1 secretion in mesangial cells treated with CSA 50 ng/mL. Addition of this analog at concentrations of 10^{-5} and 10^{-4} mol/L significantly reduced CSA-induced ET-1 secretion by 19% to 26% (Table 2).

In addition, we examined the effect of a cAMP analog, 8-bromo-cAMP, on ET-1 secretion in these cells treated with CSA 50 ng/mL. Addition of this analog at concentrations of 10^{-5} and 10^{-4} mol/L did not affect CSA-induced ET-1 secretion (Table 3).

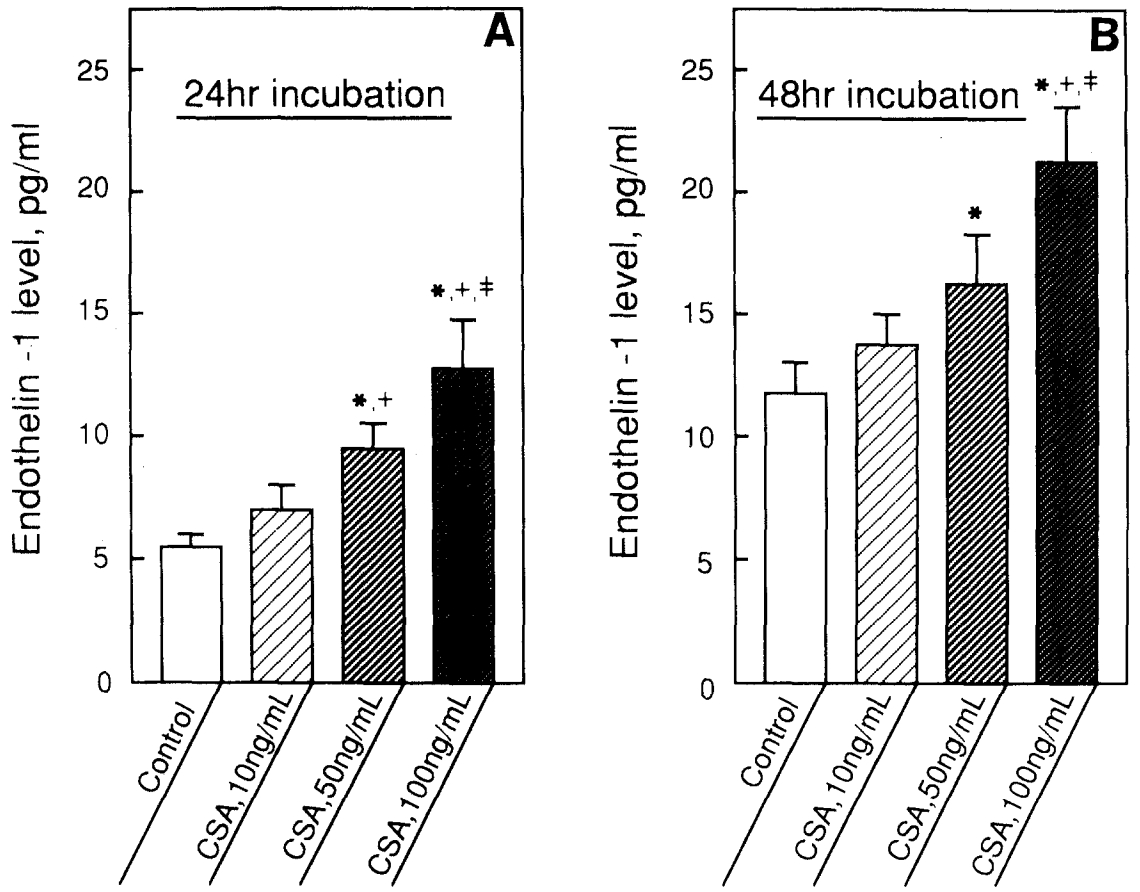

Fig 1. Effect of CSA on ET-1 secretion in cultured rat mesangial cells exposed to various concentrations of CSA (10, 50, and 100 ng/mL) for 24 and 48 hours. Each point is the mean of six measurements. *Significant difference as compared with control. †Significant difference as compared with CSA 10 ng/mL. ‡Significant difference as compared with CSA 50 ng/mL.

DISCUSSION

CSA, a fungal cyclic polypeptide, is an immunosuppressive agent widely used in organ transplantation. However, extensive use of CSA has been accompanied by potentially severe side effects, which include nephrotoxicity. In the present study, we showed that CSA in low concentrations stimulates ET-1 secretion in cultured rat glomerular mesangial cells. These findings are in agreement with previous reports that CSA stimulates ET-1 production in vivo and in vitro.[12,37-39] In contrast, CSA in high concentrations was cytotoxic and failed to stimulate ET-1 secretion in cultured rat mesangial cells. Thus, the stimulatory effect of CSA, at least at low concentration, on ET-1 secretion appears to be functional for a certain period.

Table 1. Effect of High-Dose CSA on ET-1 Secretion in Cultured Rat Mesangial Cells

	ET-1 (pg/mL)	
	24 h	48 h
Baseline	5.7 ± 0.6	11.7 ± 1.0
CSA		
10 μg/mL	5.9 ± 0.9	6.3 ± 1.7*
100 μg/mL	2.1 ± 0.4*	2.4 ± 0.5*

NOTE. Values are the mean ± SD of assays made on 6 cell cultures incubated for times indicated. Each assay was performed in duplicate.
*Significant difference as compared with baseline.

ET-1 concentrations in culture media of CSA-treated mesangial cells appear to attain levels that are within the biologically effective range for this peptide.[1] We have also shown the existence of at least two receptor subtypes, ET_A (ET-1–selective) and ET_B (equally sensitive to ET-1, ET-2, and ET-3), in cultured rat mesangial cells.[40] Furthermore, CSA is shown to increase ET-1 binding sites in cardiac smooth muscle cells[41] and may also increase binding sites in glomerular mesangial cells.[11,13] Therefore, CSA stimulates mesangial cell production of ET-1, which in turn may act on glomeruli, resulting in diminished glomerular filtration rate and renal blood flow. However, the amount of basal and CSA-induced ET-1 secretion in cultured rat mesangial cells is much lower than in cultured vascular endothelial cells. Therefore, the pathophysiologic role of ET-1 in CSA nephrotoxicity may be more important in vascular endothelial cells than in glomerular mesangial cells. Although the present in vitro study focused on the potential effect of CSA on ET-1 production, data from other studies have suggested that further mechanisms such as the prostaglandin system,[42,43] Ca^{2+} mobilization,[30] and sympathetic nerve activity[44] are implicated in CSA nephrotoxicity.

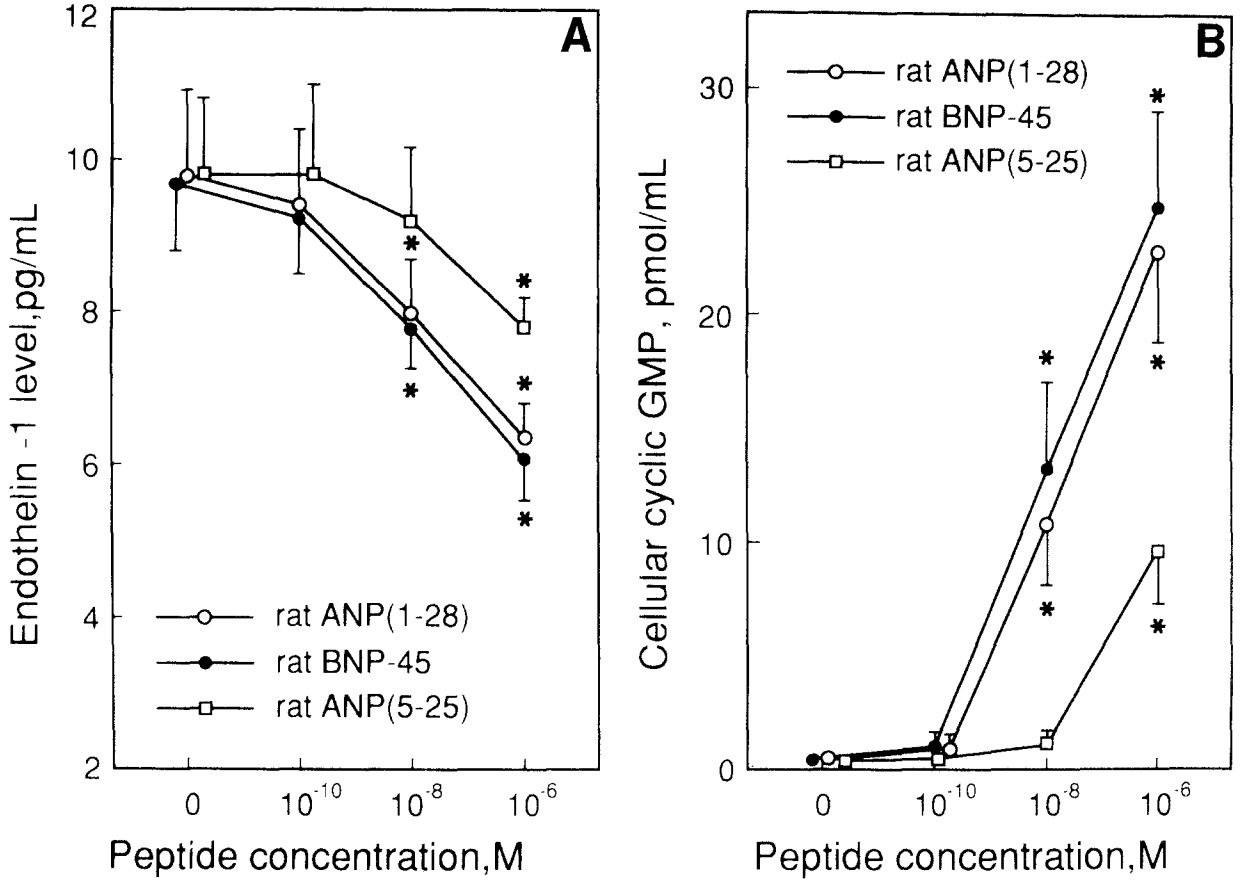

Fig 2. (A) Effects of ANP and BNP on CSA-induced ET-1 secretion in cultured rat mesangial cells exposed to different concentrations of rat ANP(1-28), rat ANP(5-25), and rat BNP-45 in addition to CSA 50 ng/mL for 24 hours. Each point is the mean of six measurements. *Significant difference as compared with CSA alone. (B) Effects of ANP and BNP on cellular cGMP level in cells treated with CSA 50 ng/mL. Mesangial cells were exposed to different concentrations of rat ANP(1-28), rat ANP(5-25), and rat BNP-45 for 30 minutes in the presence of 0.5 mmol/L IBMX. Each point is the mean of four measurements. *Significant difference as compared with CSA alone.

Next, we showed that both rat ANP(1-28) and rat BNP-45, which are the major circulating forms of ANP and BNP in rats,[27] significantly inhibited CSA-induced ET-1 secretion in cultured rat mesangial cells. Therefore, the endogenous ANP-BNP system may play a role in preventing CSA-induced nephrotoxicity through such inhibitory effects on mesangial cell ET-1 production. Actually, it is suggested that exogenously infused high-dose ANP may be useful for treatment or prophylaxis of CSA-associated side effects. Capasso et al[31] demonstrated that infusion of CSA for 7 days decreased glomerular filtration rate in rats and that this effect was completely reversed by infusion of ANP. Gianello et al[32] have also demonstrated a beneficial effect of ANP on CSA nephrotoxicity in rats. However, it remains to be clarified whether endogenous ANP and BNP have physiologic roles as modulators of mesangial cell ET-1 production in patients treated with CSA, since ANP and BNP concentrations used in this experiment are much higher than plasma levels of ANP and BNP in rats and humans.

We have obtained three pieces of evidence for a causal link between cGMP production and inhibition of ET-1 production by ANP and BNP in rat mesangial cells treated

Table 2. Effect of a cGMP Analog, 8-Bromo-cGMP, on CSA-Induced ET-1 Secretion in Cultured Rat Mesangial Cells

	ET-1 (pg/mL)
Baseline	5.6 ± 0.5
CSA 50 ng/mL	9.6 ± 1.0*
CSA 50 ng/mL + 8-bromo-cGMP 10^{-5} mol/L	7.8 ± 0.6*†
CSA 50 ng/mL + 8-bromo-cGMP 10^{-4} mol/L	7.1 ± 0.5*†

NOTE. Values are the mean ± SD of assays made on 6 cell cultures incubated for 24 hours. Each assay was performed in duplicate.
*Significant difference as compared with baseline.
†Significant difference as compared with CSA alone.

Table 3. Effect of a cAMP Analog, 8-Bromo-cAMP, on CSA-Induced ET-1 Secretion in Cultured Rat Mesangial Cells

	ET-1 (pg/mL)
Baseline	5.4 ± 0.7
CSA 50 ng/mL	9.6 ± 1.2*
CSA 50 ng/mL + 8-bromo-cAMP 10^{-5} mol/L	9.3 ± 1.1*
CSA 50 ng/mL + 8-bromo-cAMP 10^{-4} mol/L	9.4 ± 1.3*

NOTE. Values are the mean ± SD of assays made on 6 cell cultures incubated for 24 hours. Each assay was performed in duplicate.
*Significant difference as compared with baseline.

with CSA. First, rat ANP(1-28) and rat BNP-45 increased cGMP levels, and these effects paralleled inhibition of ET-1 production. Second, rat ANP(5-25) had weaker effects than rat ANP(1-28) or BNP-45 with respect to inhibiting ET-1 production and increasing cGMP in cells treated with CSA. Third, a cGMP analog reduced CSA-induced ET-1 production, whereas a cAMP analog did not affect this production. These results suggest that ANP and BNP inhibit CSA-induced ET-1 production in cultured rat mesangial cells, probably through a cGMP-dependent process.

Overall, our results suggest that low-dose CSA stimulates rat mesangial cell production of ET-1 and that cardiac hormones ANP and BNP reduce excess ET-1 production induced by CSA, probably through a cGMP-dependent process in these cells. However, inhibitory effects of these natriuretic peptides on ET-1 production in mesangial cells were weak and required much higher concentrations than their physiologic plasma levels. Furthermore, any extrapolation from experiments on cultured cells to in vivo conditions should be undertaken with some caution, because cultured cells represent at best "activated cells." Therefore, it remains to be clarified whether ANP and BNP are effective as therapeutic tools for patients treated with CSA.

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