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# ATRIAL NATRIURETIC PEPTIDE INHIBITS ENDOTHELIN-1-INDUCED ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE IN CULTURED RAT MESANGIAL CELLS

Toshiro Sugimoto, Ryuichi Kikkawa[1], Masakazu Haneda and Yukio Shigeta

The Third Department of Medicine, Shiga University of Medical Science,
Otsu, Shiga, 520-21, Japan

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**SUMMARY:** In cultured rat glomerular mesangial cells, endothelin-1 (ET-1) activated both pp 44 and pp 42 mitogen-activated protein (MAP) kinases. Atrial natriuretic peptide (ANP) inhibited ET-1-induced activation of both pp 44 and pp 42 MAP kinases. ANP also inhibited ET-1-induced translocation of protein kinase C (PKC) and TPA-induced activation of MAP kinase. These results indicate that ANP modulates the functions of mesangial cells, including proliferation and contraction through the inhibition of ET-1-induced activation of MAP kinase in various steps proximal to MAP kinase. 

Glomerular mesangial cells play an important role in the regulation of glomerular filtration rate through their contractility. Mesangial cells have receptors specific to various vasoconstrictive peptides, such as angiotensin II (1), vasopressin (2) and endothelin (3) and to vasorelaxing peptides such as ANP (4). Endothelin-1 (ET-1) is one of the most potent endogenous vasoconstrictors yet identified (5). ET-1 has been reported not only to cause the contraction but to stimulate the proliferation of the mesangial cells in vitro (3). On the other hand, ANP has been shown to counteract to contractile stimuli and to inhibit cellular proliferation in various types of cells including the mesangial cells (6). However , the mechanism of the interaction between ANP and ET-1 is still poorly understood.

MAP kinase, also known as extracellular signal-regulated kinases (ERKs), has been reported to be activated by various growth factors and vasoactive substances and suggested to play a central role in various cellular functions (7). Since ET-1 has been demonstrated to activate MAP kinase in rat mesangial cells in culture (8), ANP might

[1]To whom all correspondence should be addressed.

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inhibit the action of ET-1 in this step. To prove this hypothesis, we measured the activity of MAP kinase in cultured mesangial cells and examined the effect of ET-1 and ANP on MAP kinase. We also examined the activity of protein kinase C (PKC), which existed proximal to MAP kinase (8).

## MATERIALS AND METHODS

Materials

Rat $ANP_{1-28}$ and Endothelin-1 were obtained from Peptide Institute (Suita, Japan). Rat des- [$Gln^{18}$, $Ser^{19}$, $Gly^{20}$, $Leu^{21}$, $Gly^{22}$] -$ANP_{4-23}$-$NH_2$ (C-ANP) was purchased from Sigma (St. Louis, USA). $\gamma$ -[$^{32}$ P ] ATP (6000Ci/mmol) and [$^{3}$ H ]-phorbol 12, 13- dibutyrate (PDBu) were purchased from Dupont, NEN Research Products (Boston, MA, USA). Bovine myelin basic protein (MBP) and protein kinase inhibitor (PKI) were purchased from Sigma (St. Louis, USA). All other reagents were chemical grade and purchased from standard suppliers.

Mesangial cell culture

Glomeruli were isolated from male Sprague-Dawley rats weighing 100 to 150 g by sieving method and cultured in RPMI 1640 medium containing 20 % fetal bovine serum (FBS) and antibiotics as previously described (9). Cultured cells were identified as mesangial cells by morphological and biochemical characters as previously described (9). Cells at passage 2 to 9 were grown until 70-80 % confluency and then made quiescent by reducing the concentrations of FBS to 0.4% for 72 hr.

Measurement of the activity of MAP kinase

Quiescent mesangial cells in 60 mm dish were incubated in an incubation medium (RPMI 1640 medium / 0.4% free fatty acid (FFA) free bovine serum albumin (BSA), 20 mM Hepes, pH 7.4) containing various stimulants at 37℃ for indicated period, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed by adding 200 $\mu$ l ice-cold lysis buffer (25 mM Tris/HCl pH 7.4, 25 mM NaCl, 80 mM $\beta$-glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 1mM EGTA, 1mM PMSF, 10 $\mu$ g/ ml leupeptin). The cells were scraped off the plate and sonicated for 10 seconds. The cellular homogenates were centrifuged at 15,000 g at 4℃ for 30 min and the supernatants were used for kinase assay.

MAP kinase activity was measured by in vitro kinase assay and in situ kinase assay using bovine MBP as a substrate. In vitro kinase assay was performed according to the method described by Tobe (10) with slight modification. Assays were performed at 25 ℃ for 15 min in a final volume 40 $\mu$ l containing 25 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 1 $\mu$ M PKI, 50 $\mu$ M ATP, 2 $\mu$ Ci $\gamma$ -[$^{32}$P ] ATP, 20 $\mu$ g MBP, and 10 $\mu$ l of samples. The reactions were terminated by the addition of 10 $\mu$ l Laemmli's buffer and phosphorylated protein were separated by SDS-PAGE (7.5-12 % gradient gel ). After autoradiography to visualize phosphorylation of MBP, the activity of kinase were determined by cutting the bands and measuring radioactivity by liquid scintillation counter.

MAP kinase activity was also analyzed in situ according to method of Gotoh (11) with some modifications . Cell lysates (about 20 $\mu$ g of protein) were resolved in 10 % SDS-polyacrylamide gel containing 1mg/ml of MBP. After electrophoresis, SDS

was removed by washing with buffer A (50 mM Hepes pH 7.4 and 5 mM mercaptoethanol) plus 20 % 2-propanol. After denaturation in buffer A containing 6 M guanidine-HCl at room temperature for one hour and renaturation in buffer A with 0.04 % Tween 40 at 4 ℃ for 16 hr, the gel was incubated at 30 ℃ for one hour with 10 ml 25 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.1 mM EGTA, 5 mM 2-mercaptoethanol, 50 $\mu$ M ATP and 250 $\mu$ Ci $\gamma$ -[$^{32}$ P ] ATP for kinase reaction. The gel was extensively washed with 5 % trichloroacetic acid and 10 mM sodium pyrophosphate and then subjected to autoradiography.

[$^3$ H ]-PDBu binding to cells

Protein kinase C activation was determined by measuring the binding of [$^3$ H ]-PDBu to intact cells (12)(13). Quiescent mesangial cells in 35 mm six-well plates were incubated in binding buffer ( RPMI 1640 medium, 20 mM Hepes pH 7.4, 0.4 % BSA (FFA free)) containing agonists for 5 minutes and exposed to 5 nM [$^3$ H ]-PDBu for the last 30 seconds. Binding assays were terminated by aspirating the binding mixture and washing three times with ice-cold PBS. The cells were then solubilized with 0.5 ml of 1 N NaOH. The samples were neutralized with 0.5 ml 1 N HCl and the radioactivity associated with the cells was counted by a liquid scintillation counter. The binding of [$^3$ H ]-PDBu in the presence of 10 $\mu$ M unlabeled PDBu was considered to be nonspecific binding.

Statistical analysis

Results were expressed as Mean ± SD. Analysis of variance ( ANOVA ) with subsequent Scheffe's test was used to determine significant differences in multiple comparisons, and $p < 0.05$ was considered significant.

## RESULTS

The effect of ET-1 on MAP kinase was examined by exposing the cells to 100 nM ET-1 for 10 minutes. ET-1 increased MAP kinase activity by 3 to 4 fold over basal levels, while ANP (100 nM) did not change basal activity of MAP kinase (Fig. 1).

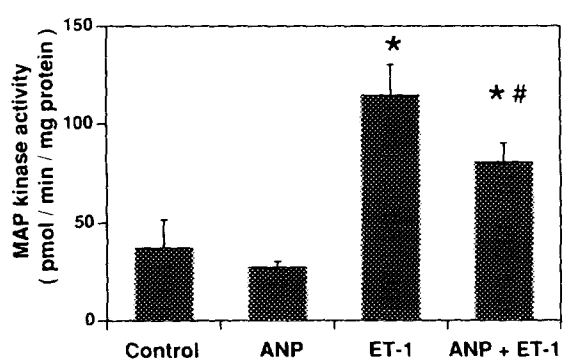
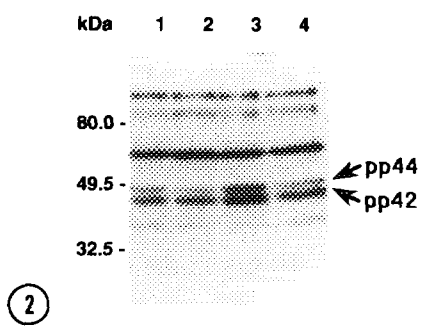


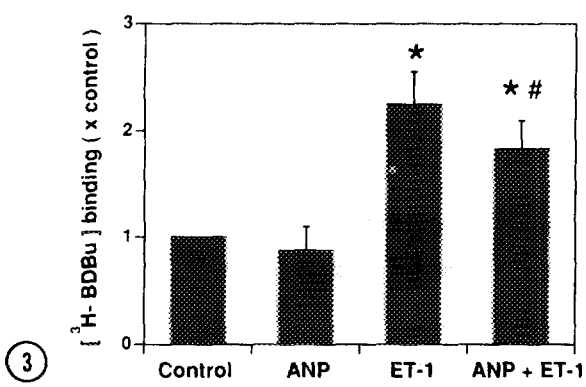

Fig. 1. Effect of ANP on ET-1-induced activation of MAP kinase in rat mesangial cells. Cells were incubated with vehicle (Control), 100 nM ANP (ANP) or 100 nM ET-1 (ET-1) for 10 min or first with 100 nM ANP for 10 min and then with 100 nM ET-1 for 10 min (ANP + ET-1). The activities of MAP kinase were measured by in vitro kinase method. Values are mean ± SD, n=6. * $p < 0.01$ vs. control, # $p < 0.01$ vs. ET-1.

When the cells were exposed to ANP 10 min prior to the addition of ET-1, ANP significantly inhibited ET-1-induced activation of MAP kinase (Fig 1). By exposing the cells to various concentrations of ANP, ET-1 (10nM)-induced MAP kinase activation was inhibited in a dose-dependent manner of ANP (% of inhibition; 9.7 ± 9.2 % at 1 nM, 12.3 ± 7.9 % at 10 nM, 51.7 ± 5.8 % at 100 nM, 62.4 ± 8.2 % at 1 $\mu$M, Mean ± SD, n=3). C-ANP (100nM), an analogue specific to the clearance receptors for ANP, failed to inhibit ET-1 (10 nM)-induced MAP kinase activation (% of basal, ET-1; 371.8 ± 56.7 %, C-ANP + ET-1; 437.9 ± 54.7 %, Mean ± SD, n=3, NS.). ET-1 activated both pp 44 and pp 42 MAP kinases ( ERK 1 and ERK 2, respectively) and ANP inhibited this ET-1-induced activation of both MAP kinases (Fig. 2).

To examine the effect of ANP on the step proximal to MAP kinase, we next examined the effect of ANP on ET-1-induced activation of PKC using PDBu binding. Increase in PDBu binding has been considered to represent an activation of PKC, because PDBu has been reported to bind predominantly to membrane-associated active form of enzyme in intact cells (12) (13). ET-1 increased the translocation of PKC by about two fold as compared with basal state. By exposure of the cells to ANP prior to ET-1, ET-1-induced translocation of PKC was significantly inhibited, though the degree of inhibition (18%) was smaller than that of MAP kinase ( Fig. 3).




(2)




(3)

Fig. 2. Measurement of MAP kinase activity in polyacrylamide gels containing MBP ( in situ kinase assay). Lane 1 : control, Lane 2 : ANP (100 nM), Lane 3 : ET-1 (10 nM), Lane 4 : ANP (100 nM) plus ET-1 (10 nM). Results are representative of three experiments.

Fig. 3. Phorbol ester ([³H]-PDBu) binding to intact rat mesangial cells. Cells were incubated with vehicle (Control), 100 nM ANP (ANP) or 100 nM ET-1 (ET-1) for 5 min or first with 100 nM ANP for 10 min and then with 100 nM ET-1 for 5 min (ANP + ET-1). Values are mean ± SD, n=9. * $p < 0.01$ vs. control, # $p < 0.05$ vs. ET-1.

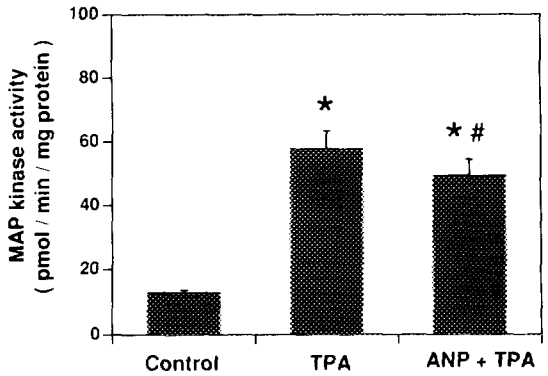



Fig. 4. Effect of ANP on TPA-induced activation of MAP kinase. Cells were incubated with vehicle (Control), 100 nM ANP (ANP) or 100 nM TPA (TPA) for 10 min or first with 100 nM ANP for 10 min and then with 100 nM TPA for 10 min (ANP + TPA). Values are mean ± SD, n=6. * $p < 0.01$ vs. control, # $p < 0.05$ vs. TPA.

To further evaluate the effect of ANP on MAP kinase activity, phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA), known to directly stimulate protein kinase C, was used as a stimulant of MAP kinase. TPA (100 nM) also caused a three to fourfold increase in MAP kinase activity (Fig. 4). ANP attenuated TPA-induced MAP kinase activation by about 15 %.

## DISCUSSION

The present study was performed to clarify the interaction between ANP and ET-1 and the results clearly indicate that ANP inhibits ET-1-induced activation of MAP kinase in cultured rat mesangial cells.

Vasoconstrictors such as ET-1 have been reported to induce the proliferation as well as contraction of cultured mesangial cells (3). As we have previously reported, vasorelaxants including ANP and nitric oxide-generating substance have anti-proliferative effect in cultured rat mesangial cells (14)(15). However, the mechanism of the anti-proliferative effect of ANP is still unknown. Recently, ET-1 has been shown to activate MAP kinase in cultured mesangial cells (8). Our results confirmed that both pp 44 and pp 42 MAP kinases were activated by ET-1 in cultured rat mesangial cells. Since MAP kinase has been reported to phosphorylate ribosomal S6 kinase II (16), c-jun (17), c-myc (18), and actin binding protein, h-caldesmon (19), MAP kinase may play an important role in the signal transduction of ET-1, and this kinase might be the target of the anti-proliferative action of ANP. To prove this hypothesis, the effect of

ANP on ET-1-induced activation of MAP kinase was examined. ANP significantly inhibited ET-1 induced activation of MAP kinase and the inhibition was observed in both pp 44 and 42 MAP kinases. Therefore, the anti-proliferative effect and, possibly, anti-contractile effect of ANP might be mediated by the inhibition of agonist-stimulated activation of MAP kinase.

The mechanism of the inhibition of MAP kinase by ANP seems to be complex. ET-1 has been reported to activate MAP kinase through the activation of PKC or the activation of unknown tyrosine kinase(s) (8). To know the mechanism of the inhibitory effect of ANP on MAP kinase, we examined the effect of ANP on both ET-1-induced translocation of PKC and TPA-induced activation of MAP kinase. ET-1-induced translocation of PKC was significantly inhibited by ANP, indicating that ANP inhibits PKC either directly, as reported in vascular smooth muscle cells (20), or by inhibiting the step(s) proximal to PKC, as suggested in cultured mesangial cells (21). However, the degree of the inhibition of ET-1-induced translocation of PKC by ANP was smaller than the inhibition of ET-1-induced activation of MAP kinase and TPA-induced activation of MAP kinase was also inhibited by ANP. These results suggest that ANP might also inhibit the step(s) distal to PKC. Therefore, the present results indicate that ANP might have diverse effects on the signal transduction system of ET-1; one, the inhibition of PKC either directly or at the step proximal to PKC and the other, the inhibition of the steps between PKC and MAP kinase. These possibilities are now under investigation in our laboratory.

In summary, the results of our study suggest that ANP inhibits MAP kinase activation and modulate cell growth and contractility in rat mesangial cells.

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