Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro

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Submitted 20 May 2002; accepted in final form 11 December 2002

Kook, Hyun, Hiroshi Itoh, Bong Seok Choi, Naoki Sawada, Kentaro Doi, Tae Ju Hwang, Kyung Keun Kim, Hiroshi Arai, Yung Hong Baik, and Kazuwa Nakao. Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro. Am J Physiol Heart Circ Physiol 284: H1388–H1397, 2003. First published December 27, 2002; 10.1152/ajpheart.00414.2002.—Both nitric oxide (NO) and natriuretic peptides produce apoptosis of vascular smooth muscle cells. However, there is evidence that NO induces endothelial cell proliferation, which suggests that there is a difference in the response of endothelial cells to natriuretic peptides. The purpose of this study was to investigate the effect of atrial natriuretic peptide (ANP) on human endothelial cell survival. ANP within the physiological concentration (10^{-11} mol/l) induced a 52% increase in the number of human coronary arterial endothelial cells and a 63% increase in human umbilical vein endothelial cells at a low concentration of serum. The increase in cell numbers was blocked by pretreatment with RP8-CPT-cGMP (RP8), a cGMP-dependent protein kinase inhibitor, with wortmannin, an Akt/PKB inhibitor, and with PD-98059, an ERK1/2 inhibitor. In a Transwell migration test, ANP also increased the cell migration, and RP8, wortmannin, and PD-98059 blocked this increase. A wound healing assay was performed to examine the effects of ANP on regeneration in vitro. ANP increased both cell numbers and migration, but the effects were blocked by the above three kinase inhibitors. ANP increased the expression of phospho-Akt and of phospho-ERK1/2 within 1.5 h. These results suggest that ANP can potentiate endothelial regeneration by cGMP-dependent protein kinase stimulation and subsequent Akt and ERK1/2 activations.

cGMP-dependent protein kinase; Akt/protein kinase B; extracellular signal-regulated kinase 1/2

Address for reprint requests and other correspondence: H. Itoh, Dept. of Medicine and Clinical Science, Kyoto Univ. Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (E-mail: hiito@kuhp.kyoto-u.ac.jp).

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reendothelialization (6), demonstrating complex responses to natriuretic peptides in different types of vascular cells. Therefore, in the present study, based on the postulation that in certain conditions natriuretic peptides may also induce an increase in endothelial cell numbers and migration, we tried to characterize the mechanism of endothelial regeneration induced by ANP. Here, we demonstrate that ANP increases cell numbers and migration by activating cGK and subsequent Akt and ERK1/2 (p44/42 MAPK) pathways. The results suggest that ANP could be useful in the regeneration of endothelial cells after injury in atherosclerosis.

METHODS

Cell cultures. Human coronary arterial endothelial cells (HCAEC; Clonetics; Waltersville, MD) and human umbilical vein endothelial cells (HUVEC; Clonetics) were grown in basic media (EBM2, Clonetics) containing growth supplements (EGM2MV or EGM2).

Cell survival. Two or three days after treatment with ANP [human ANP(1–28). Peptide Institute; Suita, Japan] or Sp-8-[4-chlorophenylthio]-guanosine 3’5’-cyclic monophosphothioate triethylamine [Sp-8-p-CPT-cGMPs triethylamine (SP8), RBI; Natick, MA], the cells were lifted with trypsin and counted with a hemocytometer. In some experiments, Rp-8-[4-chlorophenylthio]-guanosine 3’5’-cyclic monophosphothioate triethylamine [Rp-8-p-CPT-cGMPs triethylamine (RP8), RBI], wortmannin (Sigma; St. Louis, MO), PD-98059 (Calbiochem; La Jolla, CA), or SB-203580 (Calbiochem) was added 20 min before ANP treatment. Because the inhibitors can affect the normal cell growth, the inhibitor alone was treated, and the cell number when the inhibitor alone was treated was regarded as 100%.

[3H]thymidine incorporation. DNA synthesis was evaluated by [3H]thymidine incorporation as published previously (17).

Intracellular cGMP measurements. Sixteen thousand cells were seeded into each well of 24-well plates and incubated overnight. The cells were then treated with 10–11 mol/l ANP and incubated for the indicated intervals. cGMP measurements were performed according to our previous report (23) with a 125I radioimmunoassay kit (New England Nuclear; Boston, MA).

Cell migration assay using Transwell apparatus. Cell migration was measured using a Transwell migration apparatus (Costar; Cambridge, MA) with 8-μm pores coated with gelatin. Cells (2.4 × 10^6) in 120 μl EBM-0.2% BSA were loaded to the upper chamber, whereas 400 μl EBM-0.2% BSA containing ANP was loaded into the lower chamber, and incubated for 24 h at 37°C. After being stained with Diff-Quick (International Reagents; Kobe, Japan), the cells on the bottom surface were counted in six random squares of 0.5 × 0.5 mm.

Wound healing assay. The wound healing assay was performed according to a previous report (9) with some modifications. After HCAEC or HUVEC were grown to confluence in six-well plates, a scratch was made with a sterile cell scraper, and the starting point was marked by attaching a cover glass under the bottom of the plate; fresh culture media were supplied, and the cells were incubated for 3 days. The cells in the 10 small grids of an eyepiece micrometer (unit area: 0.1 mm height × 1 mm width, Olympus; Tokyo, Japan) just above the starting point were counted, and the next area from the wound side was then counted again. The cell numbers in the consecutive unit areas were counted and continued until no cells were observed. The cell counts were plotted using the consecutive field number as the abscissa, as shown in Fig. 4B, and both x- and y-axis intercepts were calculated by extrapolating the regression line.

Confocal microscopy. After treatment with ANP for 30 min, F-actin in the cells was stained with Alexa fluor 488-conjugated phalloidin (Molecular Probes; Eugene, OR) and visualized by confocal laser scanning microscopy with a Bio-Rad MRC-1024 imaging system (Hercules, CA) mounted on an Olympus Microscope (CK40) equipped with a ×60 objective lens.

Immunoblotting. After serum starvation with EBM2 containing 0.5% FBS for 24 h, HUVEC grown in a T-25 flask (Nunc; Naperville, IL) were treated with ANP and incubated further for the indicated interval. The cell lysates were prepared and blotted as in our previous report (36).

Fluorescent immunocytochemistry. After treatment with ANP for 30 min, the colocalization of both phosphoprotein and nonphosphoprotein was observed after immunocytochemistry with double antibodies as described previously (36).

RESULTS

Low-dose ANP induces an increase in endothelial cell numbers. Low-dose ANP (10−11 mol/l) increased the cell numbers of HCAEC (52.5 ± 5.5%) and HUVEC (64.2 ± 11.4%), with the increase being observed in the range of 10−13–10−11 mol/l ANP, whereas high doses (10−7–10−5 mol/l) elicited a decrease in HCAEC (Fig. 1A). A low concentration of ANP also increased [3H]thymidine incorporation, with the maximal increase being at 10−12 mol/l ANP (52.3 ± 12.2%). The increase in cell numbers induced by the low concentration of ANP became more prominent when a low concentration of serum was treated (Fig. 1B). We examined the effect of ANP on the change in the number of rat aortic smooth muscle cells cultured with 5% serum; 10−11 mol/l ANP did not affect the cell survival, whereas 10−7 mol/l ANP reduced the number of viable cells significantly (−24.6 ± 2.1%, n = 6). The following experiments were conducted using 10−11 mol/l ANP with 2% (for HCAEC) or 1% (for HUVEC) serum, which showed a prominent increase in cell numbers.

ANP-induced effects are dependent on cGK and cGMP. The basal cGMP concentration was 1.14 ± 0.25 fmol/2 × 106 cells. ANP (10−11 mol/l) increased the intracellular cGMP concentration fivefold over the basal level as early as 15 min after treatment. The increased cGMP content subsequently declined with incubation time, reaching the basal level at 12 h (Fig. 1C). A high dose of ANP (10−5 mol/l) greatly increased cGMP (77.4 ± 15.0-fold) at 15 min, followed by an abrupt decrease.

To test whether cGK is involved in the ANP-induced increase in cell numbers, the influence of cGK modulators was examined. A 2-day incubation of ANP also increased cell numbers, and the increase was completely blocked by 5 × 10−6 mol/l Rp8 (15), a cGK inhibitor, in HUVEC (Fig. 1D) and HCAEC (data not shown). Similar to the biphasic response of ANP, a low concentration (10−11 mol/l) of Sp8 (2, 15), a cGK agonist, increased cell numbers in both endothelial cell
types (Fig. 1D), whereas a high dose ($10^{-5}$ mol/l) decreased the number by $-23.3 \pm 6.1\%$ (HCAEC) and $-35.2 \pm 13.0\%$ (HUVEC).

Protein kinases are involved in ANP-induced effects. We investigated the subsequent signal transduction pathway mediating the ANP-induced increase in endothelial cell numbers by pretreatment with the inhibitors of Akt, ERK1/2, and p38 MAPK (stress-activated protein kinase 2). Concentrations that minimally affected the basal cell number were selected (data not shown): $10^{-7}$ mol/l wortmannin, $10^{-5}$ mol/l PD-98059, and $5 \times 10^{-6}$ mol/l SB-203580 (12, 15, 31, 45). Pretreatment with either wortmannin or PD-98059 significantly blocked the increase induced by $10^{-11}$ mol/l ANP, whereas the administration of SB-203580 showed no significant change (Fig. 2A). The inhibition induced by wortmannin or PD-98059 was dose dependent (Fig. 2, B and C).

Low-dose ANP provokes migration. First, we examined the effect of ANP on the migration with a conventional Transwell apparatus. Compared with the control, the numbers of HUVEC that migrated into the lower surfaces of the membranes increased in the ANP-treated group: $10^{-11}$ mol/l ANP increased cell
migration by as much as 2.5 times \( P < 0.01 \). bFGF, a positive control, increased migration \( P < 0.01 \); Fig. 3A). Low-dose \( 10^{-11} \) mol/l SP8 simulated the ANP effect (Fig. 3A). In contrast, pretreatment with RP8 blocked the increase in migration. Pretreatment with either wortmannin or PD-98059 significantly inhibited the migration. However, SB-203580 did not significantly affect migration (Fig. 3B).

Wound healing assay. In the wound healing assay, the total number of cells that migrated into the cell-
free area 3 days after the wound was made was counted and plotted as shown in Fig. 4A. ANP (10^{-12} mol/l) increased the total number of migrated cells in the cell-free area.

The cells in the wound area could be divided into two different origins: cells that mainly migrated from the wound margin (migration component) and cells that mainly underwent mitosis (proliferation component). Therefore, we counted the cells in the small unit areas, plotted as seen in Fig. 4B, and calculated the x- and y-axis intercepts by extrapolation, which can represent, to a certain degree, the extent of migration and proliferation, respectively. ANP as well as 10 ng/ml VEGF significantly increased the migrating extent. The increase in migration induced by ANP was completely blocked by pretreatment with RP8 (Fig. 4C). ANP also significantly increased cell density at the starting point, a proliferating component, and, again, the increase was completely blocked by pretreatment with RP8 (Fig. 4D).

Pretreatment with either wortmannin or PD-98059 also attenuated the ANP-induced increase in total cell count (Fig. 5A). ANP-induced increases in the x-axis intercepts as well as the y-axis intercepts were blocked by pretreatment with either wortmannin or PD-98059. SB-203589 failed to block the ANP-induced increase in both intercepts (Fig. 5, B, C, and D).

Changes in actin arrangement. In untreated HUVEC, F-actin was found mostly in cortical structures (Fig. 6A). ANP produced reorganization of F-actin, characterized by the formation of long stress fibers that transversed the cells (Fig. 6B) and by the formation of membrane ruffling (Fig. 6C). However, the actin rearrangement was completely blocked by pretreatment with RP8 (Fig. 6D).

ANP induces phosphorylation of Akt and ERK1/2. After treatment with ANP, the contents of phospho-Akt and phospho-ERK1/2 were increased, and such increases were prominent within 1 h after treatment. However, the nonphosphorylated (total) Akt and ERK1/2 were unaltered by ANP treatment (Fig. 7, A and B).

Visualization of phospho-Akt and phospho-ERK1/2. In ANP-treated groups, some cells showed strong positive green fluorescence with phospho-Akt (Fig. 7G), whereas red fluorescence indicating Akt was not significantly altered (Fig. 7H), implying an increase in cell

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Fig. 4. The wound healing assay and its interpretation showing the proliferation and migration phases of migrated cells. A: total cell numbers that migrated into the cell-free area 3 days after the wound was made. VEGF served as a positive control. B: cell counts in consecutive areas from the wound margin. The cell numbers in each unit area were counted, plotted, and extrapolated to divide the migration component (x-axis intercepts) and proliferation component (y-axis intercepts). C: x-axis intercepts obtained from B, which show the extent of migration of cells. D: y-axis intercepts, which indicate virtual cell densities at the wound starting points. Values are means ± SE from 9 cases. **P < 0.01, significant difference from control; @P < 0.05 and @@P < 0.01, significant differences from the ANP-treated group.
numbers with phosphorylated Akt. ANP also increased the phosphorylation of ERK1/2 (Fig. 7M).

DISCUSSION

This study documents a novel role of ANP in promoting endothelial regeneration. We showed that migration, as well as the number of endothelial cells, is increased by a low concentration of ANP and that both Akt and ERK1/2 pathways play principal roles in the signal transduction of ANP effects. We used a wound healing assay as an in vitro experimental model, and the results were consistent with our previous observations: that natriuretic peptides induce reendothelialization in an experimental animal model (6).

A series of studies have revealed that NO is a key molecule that induces vascular endothelial cell growth (46) and that it mediates VEGF-induced endothelial cell proliferation and migration (27). It has been proposed that increased cGMP, a downstream molecule of NO signal transduction, and subsequently activated cGK mediate the VEGF-induced MAPK activation in endothelial cells (15, 31). Thus the evidence that the cGMP/cGK system is related to vascular endothelial cell proliferation and migration raises the possibility that natriuretic peptides can possess similar potency on endothelial cells, because they share the same intracellular signal transduction pathway as NO.

In the present study, especially when the lower concentration of ANP within the physiological range (28, 40) was added, ANP increased cell numbers as well as DNA synthesis, suggesting the increase in cell numbers is caused by proliferation in part, although other mechanisms, such as the inhibition of apoptosis, may be involved simultaneously. In addition, the physiological concentration of ANP increased cell migration in both a Transwell migration test and wound healing assay. To our knowledge, this is the first demonstration of a natriuretic peptide-induced increase in cell numbers and migration in cultured human endothelial cells in an in vitro model, although one report mentioned that natriuretic peptide is associated with a higher cell growth rate in bovine bone endothelial cells (4).
that natriuretic peptides inhibit cell growth in the
concentration is shown. Lower concentration, whereas a decrease at a higher
factors in inducing an increase in cell numbers at a
ANP also seems to follow the same pattern of growth
and proliferation (42) in a bell-shaped fashion. Thus
many growth factors also induced migration (25, 35)
were blocked by RP8. Furthermore, SP8, a cGK acti-
migration and proliferation of the endothelial cells
was increased by low-dose ANP but also the increased
serum, which might have caused masking of the ANP-
induced cell proliferation. We observed an increase in
cells only in groups treated with a low concen-
tion of serum (5%) without any growth factors
were related to the increase in intracellular cGMP and
the activation of cGK and strongly suggest that cGMP
and cGK are involved in the ANP effects, although
there might be mechanisms other than cGMP/cGK.

In the present study, ANP showed a typical bell-
shaped response in the increase in cell numbers and
migration, and the cGMP analog also induced biphasic
changes: an increase with low doses but a decrease
with high doses. High concentrations of ANP did not
increase cell numbers, even though an increase in
cGMP became much more prominent. It has been re-
peatedly shown that the effects of many growth factors
do not follow a dose-dependent sigmoidal curve but a
bell-shaped response, although the mechanism is not
fully understood (14, 29, 30, 32). In endothelial cells,
many growth factors also induced migration (25, 35)
and proliferation (42) in a bell-shaped fashion. Thus
ANP also seems to follow the same pattern of growth
factors in inducing an increase in cell numbers at a
lower concentration, whereas a decrease at a higher
concentration is shown.

Early studies, including our own report, have shown
that natriuretic peptides inhibit cell growth in the
endothelial cell obtained from rats (16) and bovines
(18). However, in those studies, the experimental con-
ditions differed from ours in several points: the higher
concentration of natriuretic peptides, different serum
concentration, phenotypic alteration by subculture
(39), and the species of animals employed. In those
reports that showed growth arrest by natriuretic pep-
tides, an inhibitory effect was observed at high concen-
trations of natriuretic peptide, such as $10^{-8}$–$10^{-6}
mol/l. In our present study, we also observed a decrease
in cell numbers at such concentrations. Considering
that these concentrations are much higher than phys-
iological conditions, as we reported (28, 40), the inhib-
itory response of cell growth does not seem to be of
physiological significance. Moreover, their experiments
were performed with a relatively high concentration
of serum, which might have caused masking of the ANP-
induced cell proliferation. We observed an increase in
cell numbers only in groups treated with a low concen-
tration of serum (<5%) without any growth factors
included. Increasing the serum concentration abruptly
decreased the ANP-induced increase in cell numbers.
These observations suggest that the effects of prolifera-
tive stimuli could be dampened by strong mitogens. A
report that CNP reciprocally antagonizes the mito-
genic signaling of serum in fibroblasts (3) supports our
present data, showing that cell proliferation is promi-
nent only at a low concentration of serum.

Cell migration by extracellular mitogenic signals has
been shown to be associated with alteration of actin
reorganization into stress fibers (34, 41). Actin reorga-
nization is characterized by the formation of lamellipo-
dia, a transverse arrangement of stress fibers, mem-
brane ruffling, and a forward extension in direction of
movement (37). We found that ANP mediates stress
fiber formation, consistent with the results of increased
migration. Stress fiber formation and increased migra-
tion were blocked by RP8, suggesting the involvement
of cGK in ANP-induced endothelial cell motility. These
results are quite opposite to previous reports in which
the natriuretic peptide family inhibits serum-induced
endothelial cell migration (16) and lipid-induced vas-
cular smooth muscle cell migration (20). However, as
described above, they observed the effects at higher
concentrations of natriuretic peptides than ours and
under mitogen-stimulated conditions.

Members of the MAPK family, including ERK1/2 and
p38 MAPK, are important mediators of signal trans-
duction generated by growth factors, cytokines, and
stressing agents, thereby regulating cellular growth
and migration (33). ERK1/2 stimulates several factors,
c-Fos, Elk-1, etc., thereby initiating DNA synthesis and
proliferation (44). Another protein kinase, serine/threo-
in protein kinase Akt/PKB, which is activated
by phosphoinositide 3′-kinase (11), is increasingly rec-
ognized as a key regulator of cell growth and migration
(8, 19). The involvement of the Akt signaling pathway
in endothelial cell proliferation and migration has also
been elucidated (12, 26). p38 MAPK, also referred as
stress-activated protein kinase 2, mediates actin reor-
ganization and cell migration in human endothelial

![Fig. 6. ANP-induced stress fiber formation. After treatment with ANP, F-actin was stained with Alexa fluor 488-conjugated phalloidin and visualized with confocal laser scanning microscopy. A: control. B and C: ANP-treated cells. D: RP8-pretreated cells.](image-url)
cells induced by mitogens such as VEGF (34) or by platelet-derived growth factor (25). Therefore, we explored the signal transduction pathways of the ANP-induced events, focusing on Akt, ERK1/2, and p38 MAPK as possible intracellular mechanisms. In the present study, we demonstrated that the ANP-induced increases in both cell number and migration were blocked by either wortmannin or PD-98059, suggesting the involvement of Akt and ERK1/2 pathways in the ANP effects. In addition, the protein contents of both phospho-Akt and phospho-ERK1/2 were significantly increased in the early phase of ANP treatment. However, p38 MAPK did not seem to be involved in the ANP-mediated endothelial cell migration. The early increases in these phosphoprotein contents, as well as the cGMP level, seemed to trigger proliferation.

Raf-1, an initiating molecule in growth factor-induced proliferation, might play a key role in mediating the ANP/cGMP/cGK signal to MAPK pathways, because cGK directly associates with the Raf-1 and thereby activates the cascade involving ERK1/2 in human endothelial cells (15). It has been clearly elucidated that Akt activates endothelial NO synthase by phosphorylation, resulting in the increase in cGMP (5, 13). Conversely, Li et al. (24) found that reagents increasing intracellular cGMP levels activate Akt in primary hepatocytes and that wortmannin completely inhibited cGMP-induced Akt activation. These results suggest that phosphatidylinositol 3-kinase and Akt may be stimulated by cGMP, supporting our observations that activated cGK by ANP may play a role in endothelial cell growth through cytoprotective Akt pathways. Activated Akt also mediates the endothelial cell migration by forming stress fibers and by actin reorganization (26), implying that the increased migration induced by ANP in the present study is partly mediated by Akt.

In summary, we demonstrated that ANP increases both cell number and migration, that the effects are prominent with a physiological concentration of ANP, and that the resulting increase in cGMP and activated...
cGK are responsible for the activation of Akt and ERK1/2 pathways. The finding that these ANP mediate endothelial cell proliferation and migration by activating Akt and ERK1/2 signal transduction identifies natriuretic peptides as a new target to modulate angiogenesis. In this context, natriuretic peptides or their downstream molecules may have interesting therapeutic potential for inducing endothelial regeneration after vascular injury.

The authors express thanks to Dr. Young Do Jung of the Department of Biochemistry, Chonnam National University Medical School, for critical comments.

H. Kook was supported by a grant from the Chonnam National University Research Institute of Medical Sciences.

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