Knockdown of natriuretic peptide receptor-A enhances receptor C expression and signalling in vascular smooth muscle cells

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Aims

Natriuretic peptide receptor-A (NPR-A) knockout mice exhibited an increased blood pressure that may also be attributed to the up-regulation of NPR-C and associated signalling; however, the interaction between the two receptors has not been investigated. In the present study, we investigated the effect of knockdown of NPR-A using NPR-A antisense (AS) on the expression of NPR-C and adenylyl cyclase (AC) signalling in A10 vascular smooth muscle cells (VSMC).

Methods and results

The receptor and G protein expression was determined by western blotting, and AC activity was determined by measuring [32P]cAMP formation from [α-32P]ATP. Treatment of A10 VSMC with NPR-A AS decreased NPR-A and enhanced NPR-C expression without altering the levels of angiotensin II AT1 and muscarinic M2 receptors. In addition, siRNA-NPR-A also resulted in the up-regulation of NPR-C. The re-expression of NPR-A in AS-treated cells reversed the enhanced expression of NPR-C to control levels. In addition, NPR-C-, AT1, and M2 receptor-mediated inhibition of AC and Giα protein expression was enhanced in AS-treated cells, whereas NPR-A-mediated cyclic GMP (cGMP) formation and Gsα-mediated stimulation of AC were significantly reduced. Pertussis toxin treatment attenuated the AS-induced enhanced inhibition of AC to control levels. Furthermore, the enhanced levels of NPR-C and Giα proteins were reversed to control levels by 8-bromo-cGMP (8Br-cGMP) and PD98059, an MEK inhibitor. In addition, 8Br-cGMP also attenuated AS-induced enhanced ERK1/2 phosphorylation to control levels.

Conclusion

These results demonstrate that knockdown of NPR-A up-regulates the expression of NPR-C, Giα proteins, and NPR-C-linked AC signalling and suggests a cross-talk between NPR-A and NPR-C.

Keywords

NPR-A/NPR-C • NPR-A antisense/siRNA • Gi protein • Adenylyl cyclase • VSMC

1. Introduction

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) belong to the family of natriuretic peptides (NP)1,2 and are produced in mammalian hearts, including humans.3,4 ANP, through its interaction with receptors on the plasma membrane and altering the levels of second messengers such as cAMP, cyclic GMP (cGMP), inositol turnover, or ion channels, regulates a variety of physiological functions;5,6 including blood pressure.

Natriuretic peptide receptors (NPR) are divided into two major categories, NPR-A/NPR-B7,8 and NPR-C.9,10 NPR-A and NPR-B are membrane guanylyl cyclase receptors with a molecular mass of ~120 kDa. NPR-A preferentially binds ANP and BNP, whereas NPR-B is more selective for CNP. On the other hand, two different subtypes of NPR-C with a molecular mass of 67 and 77 kDa have been identified with a broad range of ligands, including ANP, BNP, CNP, and C-ANP4–23[des(Gln18, Ser19, Gly20, Leu21, Gly22)]11; however, C-ANP4–23 is specific for NPR-C and has no affinity for NPR-A or NPR-B.11,12 The 77 kDa protein is implicated in ligand internalization as a clearance receptor13 and has a low affinity for CNP, whereas the 67 kDa protein shows a high affinity for CNP.14 The 67 kDa protein is coupled to adenylyl cyclase (AC) inhibition through inhibitory guanine nucleotide regulatory protein G10,15 or to activation of phospholipase C.16 NPR-C and cAMP signal transduction has been shown to mediate several physiological functions that include inhibition of endothelial and vascular smooth muscle proliferation, inhibition of...
adrenergic and purinergic neurotransmission, in vivo translation of the endothelin message, the secretion of endothelin from cultured bovine aortic endothelial cells, inhibition of progesterone secretion from Leydig tumour cells, and inhibition of thyroglobulin release from cultured human thyroid cells. In addition, a role of NPR-C (67 kDa protein) and AC inhibition by water deprivation has also been reported as one of the mechanisms through which NP regulate kidney functions.13

The expression of NPR-C and signalling has been shown to be regulated by several hormones whose levels are augmented in hypertension.17–20 We have previously shown that angiotensin II (Ang II), vasopressin, endothelin, C-ANP4–23, and ANP down-regulated the expression of NPR-C in vascular smooth muscle cells (VSMC),17–20 which was associated with the attenuation of NPR-C-mediated inhibition of AC. Furthermore, Kishimoto et al.21 reported that the treatment of VSMC with NP such as ANP, BNP, and CNP resulted in the decreased expression of NPR-C. In addition, nitric oxide and cGMP, a second messenger of NPR-C was regulated by several hormones whose levels are augmented in vascular smooth muscle cells (VSMC),17–20 whereas cAMP a second messenger of NPR-C was shown to down-regulate the expression of NPR-B and up-regulate the expression of NPR-C in FRTL-5 rat thyroid cells,25 and human aortic smooth muscle cells, respectively.26 However, the effect of knocking down or knocking out of one NP receptor subtype on the expression of other NP receptor subtype and associated cell signalling has not been explored.

We have previously shown that ablation of NPR-C by NPR-C antisense (AS) oligonucleotide resulted in enhanced stimulation of cGMP levels (second messenger of NPR-A activation) by ANP.27 Furthermore, NPR-C knockout mice lacking the Npr3 gene also showed increased cGMP excretion and decreased blood pressure.28 These data suggest that a cross-talk exists between NPR-C and NPR-A; however, this possibility has not been explored. Similarly, NPR-A knockout mice have been reported to exhibit an increased blood pressure.29 However, the contribution of NPR-C and associated signalling in this response has not been investigated. The present study was therefore undertaken to examine the effect of knocking down of NPR-A by AS oligonucleotides or siRNA of NPR-A on the expression of NPR-C and associated signalling in A10 VSMC. We demonstrate for the first time that knockdown of NPR-A up-regulates the expression of NPR-C, Gix proteins, and NPR-C-linked AC signalling and suggests a cross-talk between NPR-A and NPR-C.

2. Methods

2.1 Materials

[α-32P]ATP was from Amersham. C-ANP4–23 was from Calbiochem and the antisense oligodeoxynucleotide sequence (AS, GTT CTC CCC ATC AGT AAG AGT TC; sense, GAA CTC TTA CTG ATG GGG AGA AC) was purchased from Alpha DNA. Small interfering RNA (siRNA)-NPR-A and antibodies were purchased from Santa Cruz biotechnology. All other chemicals were obtained from Sigma-Aldrich. The AS 21-mer phosphorothioate oligodeoxynucleotide (OH-2) used was against a sequence of nucleotides specific to the NPR-A.

2.2 Cell culture and incubation

VSMC line (A10) from the rat embryonic thoracic aorta was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics and 10% foetal calf serum (FBS) as described previously.27 The confluent cells were incubated in the absence or presence of 5 μM (or as otherwise indicated) sense or AS oligonucleotides for 48 h at 37°C as described in detail in the Supplementary material online.

2.3 Knockdown and re-expression of NPR-A in A10 VSMC

Confluent cells were incubated with or without 5 μM, NPR-A AS for 6 h and were supplemented with 10% FBS and incubations were continued for 48 h. After incubation, both control and AS-treated cells were trypsinized and re-plated at 50% confluency and were grown overnight at 37°C. The cells were transfected with pCMV6-NPR-A [NM_000996.2, OriGene Technologies (Rockville, MD, USA)], expressing full-length human NPR-A, and for transient overexpression, pCMV6-GFP, expressing green fluorescent protein, was used in control transfections.

2.4 Transfection of cells with siRNA of NPR-A

Confluent cells were incubated in serum-free medium for 5 h and were transfected with siRNA-NPR-A (250 nM) using Lipofectamine 2000 (Invitrogen, CA, USA) for 48 h. After the incubation, the cell lysates were prepared and used for western blotting. A scramble siRNA was used as a negative control.

2.5 Western blotting

The levels of NPR-A, NPR-C, and Gix proteins were determined by western blotting using specific antibodies as described in detail in the Supplementary material online.

2.6 Determination of AC activity

AC activity was determined by measuring [32P]cAMP formation from [α-32P]ATP, as described previously.10,17 Briefly, the assay medium containing 50 mM glycyglycine, pH 7.5, 0.5 mM MgATP, [α-32P] ATP (1.5–10) × 105 c.p.m., 5 mM MgCl2 (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM IBMX, 0.1 μM EGTA, and 10 μM GTPγS and an ATP-regenerating system consisting 2 mM phosphocreatine, 0.1 mg of creatine kinase/mL, and 0.1 mg of myokinase/mL in a final volume of 200 μL were pre-incubated for 2 min at 37°C. The reaction was initiated by the addition of the membrane proteins (20–30 μg) to the reaction mixture. The reactions were terminated after 10 min by the addition of 0.6 mL of 120 mM zinc acetate. cAMP was purified by co-precipitation of other nucleotides with ZnCO3, by addition of 0.5 mL of 144 mM Na2CO3, and subsequent chromatography by the double-column system, as described by Salomon.30

2.7 Determination of intracellular cGMP levels

Intracellular cGMP levels were determined as described previously.27 After pre-incubation with AS or sense oligonucleotide, the cells were washed twice with phosphate-buffered saline. The cells were further incubated with 1 mM IBMX for 5 min at 37°C and stimulated with or without ANP1–28 (28 amino acids, positions 99–126, of ANP) at various concentrations for 10 min. The reaction was terminated by rapid aspiration and addition of ice-cold 1 M HCl. cGMP levels were determined by radioimmunoassay using a radioimmunoassay (RIA) kit from Biomedical Technologies (Stoughton, MA, USA).

2.8 Statistical analysis

Results are expressed as the mean ± SEM. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Results were considered significant at a value of P < 0.05.
3. Results

3.1 NPR-A AS oligonucleotide attenuates the expression of NPR-A and NPR-A-mediated cGMP levels and up-regulates NPR-C protein expression

Treatment of A10 VSMC with NPR-A AS oligodeoxynucleotide for 48 h resulted in the attenuation of NPR-A expression as determined by western blotting using NPR-A-specific antibodies. The expression of NPR-A protein was significantly reduced by ~60% in cells treated with 5 μM AS oligonucleotide compared with control cells; however, at 2.5 μM, the decrease was ~20%, and at 1 μM, the levels of NPR-A were not altered (Figure 1A). On the other hand, the treatment of cells with sense oligonucleotide did not alter the levels of NPR-A protein (Figure 1B). In addition, the reduction in the expression of NPR-A protein by 5 μM AS oligonucleotide was associated with decreased formation of cGMP levels by ANP₁₋₂₈. As shown in Figure 1C, ANP₁₋₂₈ increased the levels of cGMP in a concentration-dependent manner in control cells, a maximal increase of ~180% was observed at 1 μM; however, this increase was completely abolished by AS treatment. The AS treatment of cells also decreased the basal levels of intracellular cGMP by ~50% (12.8 vs. 28.2 pmol cGMP/mg of protein).

In addition, the treatment of cells with 5 μM AS for 48 h increased the expression of NPR-C by ~70%; however, at 1 and 2.5 μM, the increase was ~10 and 20%, respectively, as determined by densitometric scanning (Figure 1D). On the other hand, the treatment of cells with 5 μM sense did not alter the levels of NPR-C (Figure 1E). In addition, the increase in the levels of...
NPR-C by AS treatment was time-dependent and associated with a concomitant decrease in the levels of NPR-A as shown in Figure 2. About 20–30% increase in NPR-C was observed at 24 h, whereas 48 h of AS treatment augmented the expression by \( \approx 75\% \) (Figure 2A). This increase in NPR-C was accompanied by a decrease in the expression of NPR-A by \( \approx 20–30\% \) at 24 h and \( \approx 70\% \) at 48 h of treatment (Figure 2B).

### 3.2 Effect of siRNA of NPR-A on NPR-C protein expression

To further confirm the cross-talk between the two receptors, siRNA approach was used to examine the effect of NPR-A silencing on the expression of NPR-C protein in A10 cells. Results shown in Figure 2C indicate that the transfection of cells with NPR-A siRNA decreased the expression of NPR-A by \( \approx 60\% \) with a concomitant increase (−50 to 60%) in the levels of NPR-C protein (Figure 2D).

### 3.3 NPR-A AS does not alter the expression of Ang II AT1 and muscarinic M2 receptors

Since AT1 and M2 are also Gi-coupled receptors\(^{31,32}\) and upon activation inhibit AC activity, it was of interest to examine whether NPR-A AS treatment could also augment the expression of these receptors. Results shown in Figure 2E and F indicate that the expression of AT1 (Figure 2E) and muscarinic M2 (Figure 2F) receptors exhibited by A10 cells was not altered by NPR-A AS (5 \( \mu M \)) treatment for different periods of time (12–48 h) as determined by western blotting.
3.4 Re-expression of NPR-A in AS-treated cells restores the enhanced expression of NPR-C

To further confirm the mutual reciprocal relationship between NPR-A and NPR-C, the effect of transient overexpression of NPR-A on the expression of NPR-C was examined in AS-treated cells. Results shown in Figure 3 indicate that the treatment of cells with AS reduced the NPR-A protein level by \( \approx 75\% \) in comparison to untreated control cells (Figure 3B) which was accompanied by an increase in NPR-C expression by \( \approx 60\% \) (Figure 3A). Furthermore, transient overexpression of NPR-A in NPR-A AS-treated cells resulted in the restoration of enhanced expression of NPR-C to the control levels (Figure 3A), whereas the levels of NPR-A protein was increased by two-fold (Figure 3B). On the other hand, transient overexpression of NPR-A in GFP expressing control cells increased the NPR-A protein levels by more than six-fold (Figure 3B) and decreased the levels of NPR-C protein by 50% (Figure 3A).

3.5 Up-regulation of NPR-C by AS treatment reflects in enhanced NPR-C-mediated inhibition of AC

To investigate whether the augmentation of NPR-C expression by NPR-A AS is reflected in NPR-C-mediated functions, the effect of AS treatment on AC inhibition by C-ANP\(_4\)–\(23\) was studied in A10 VSMC. Figure 4A shows that C-ANP\(_4\)–\(23\) inhibited AC activity in a concentration-dependent manner in control and sense- and AS-treated A10 cells. However, the inhibitory effect of C-ANP\(_4\)–\(23\) was significantly augmented by \( \approx 120\% \) (25 vs. 55%) in cells treated with AS, but not in cells treated with sense oligomer.

3.6 NPR-A AS augments Ang II- and oxotremorine-mediated inhibition of AC activity

Since the expression of AT1 and M2 receptors is not altered by AS treatment, it was of interest to investigate whether AS treatment was also ineffective in altering AT1 and M2 receptor-mediated inhibition of AC. The results shown in Figure 4B indicate that Ang II and oxotremorine inhibited AC activity in both control and AS-treated cells; however, the inhibition was significantly enhanced in AS-treated cells by about two-fold.

3.7 NPR-A AS augments the Gi protein levels

Since the AS treatment of VSMC enhanced the extent of inhibition of AC by Ang II and oxotremorine without increasing the expression of AT1 and M2 receptors, it may be possible that post-receptor components may also be altered by AS treatment and contribute to the enhanced response. To investigate this possibility, the levels of Gi\(_a\) proteins that couple AT1 and M2 receptors to AC were determined by the western blotting technique using specific antibodies against Gi\(_a\)-2 and Gi\(_a\)-3 at different times of pre-treatment with AS. Results shown in Figure 5 indicate that the levels of Gi\(_a\)-2 (Figure 5A) and Gi\(_a\)-3 (Figure 5B) (Gi\(_a\)-1 is absent in the aorta) were not altered at 12 and 24 h of pre-treatment with AS; however, pre-treatment for 48 h increased the levels by \( \approx 40\text{–}50\% \) as determined by densitometric scanning. These results suggest that the augmentation of C-ANP\(_4\)–\(23\)-mediated inhibition of AC by two-fold at 48 h of pre-treatment (Figure 4A) may be attributed to the increased levels of Gi\(_a\) proteins as well as to the up-regulation of NPR-C. In addition, the levels of Gs\(_a\) were not altered by AS treatment (data not shown).
3.8 Effect of NPR-A AS on Gi functions
To investigate whether the increased levels of Giα were also reflected in Gi functions, the effect of low concentrations of GTPγS on forskolin (FSK)-stimulated AC activity (a marker for receptor-independent Gi functions) was examined in control and AS-treated cells. Figure 5C illustrates that GTPγS inhibited FSK-stimulated AC activity in control and AS-treated cells in a concentration-dependent manner; however, the inhibition was significantly augmented in AS-treated cells by ≏60% (≏70 vs. 45%). On the other hand, AS treatment also decreased the basal AC activity by ≏30% (180.2 ± 30.7 vs. 128.2 ± 46.5 pmol cAMP/10 min/mg of protein) as well as FSK-stimulated activity by ≏20% (42-fold stimulation vs. 35.5-fold over basal).

3.9 Pertussis toxin treatment attenuates Gi-mediated AC hormonal inhibition in control and NPR-A AS-treated cells
To investigate whether the enhanced inhibition of AC by C-ANP4–23, Ang II, and oxotremorine is attributed to the enhanced expression of Giα proteins, the effect of pertussis toxin (PT), which inactivates and decreases the levels of Giα proteins,34 on C-ANP4–23, Ang II, and oxotremorine-mediated inhibition of AC activity was examined in control and AS-treated cells. Results shown in Figure 5D illustrate that the enhanced inhibition of AC by C-ANP4–23, Ang II, and oxotremorine was attenuated to control levels in AS-treated cells by PT treatment. In addition, PT also attenuated the inhibition exerted by these hormones in control cells.

3.10 NPR-A AS attenuates Gs-mediated stimulation of AC
In order to investigate whether AS treatment also results in the attenuation of other receptor-mediated AC signalling, the effect of AS treatment on Gs-mediated stimulation of AC by isoproterenol, glucagon, and FSK was examined. The results shown in Figure 5E indicate that isoproterenol, glucagon, and FSK stimulated AC activity by 30, 45, and 4230%, respectively, in control cells; however, these stimulatory effects were significantly diminished by ≏25, 20, and 25%, respectively, by AS treatment.

3.11 Decreased levels of intracellular cGMP due to NPR-A knockdown contribute to up-regulation of NPR-C and Giα proteins through MAP kinase signalling pathway
To investigate whether the decreased levels of intracellular cGMP due to NPR-A knockdown contribute to up-regulation of NPR-C and Giα proteins through MAP kinase signalling pathway, we examined the effect of 8-bromo-cGMP (8Br-cGMP) on the expression of NPR-C and Giα proteins in control and AS-treated cells. Results shown in Figure 6A indicate that AS-induced increased expression of NPR-C was reversed towards control levels by ≏70% by 8Br-cGMP. In addition, 8Br-cGMP, as reported earlier,23 also decreased the levels of NPR-C in control cells by ≏35%. Furthermore, the increased expression of Giα-2 (Figure 6B) and Giα-3 (Figure 6C) induced by AS was also reversed to almost control levels by 8Br-cGMP. In addition, as reported previously,23 8Br-cGMP also decreased the expression of Giα proteins by ≏20% in control cells.
Since MAP kinase pathway has been implicated in the decreased expression of NPR-C and Giα proteins induced by nitric oxide, which increases the intracellular levels of cGMP in VSMC, it was of interest to examine whether MAP kinase pathway contributes to the increased expression of NPR-C and Giα proteins induced by NPR-A AS. To test this, we examined the effect of PD98059, an inhibitor of MEK, on the expression of NPR-C and Giα proteins induced by NPR-A AS. Results shown in Figure 6B indicate that the AS-induced enhanced expression of NPR-C (Figure 6D) and Giα proteins (Figure 6E and F) was attenuated by PD98059. To further investigate whether NPR-A AS treatment could also increase the activity of MAP kinase due to the decreased levels of intracellular cGMP, we examined the effect of NPR-A AS on ERK1/2 phosphorylation in the absence and presence of 8Br-cGMP. Results shown in Figure 6F indicate that ERK1/2 phosphorylation was significantly enhanced in AS-treated cells, which was reversed to control levels by 8Br-cGMP.

4. Discussion

We demonstrate for the first time that knockdown of NPR-A by using AS oligonucleotide and siRNA of NPR-A results in the overexpression of NPR-C in A10 VSMC, suggesting a cross-talk between the two receptor subtypes of NPR family.
We showed that the treatment of VSMC with NPR-A AS attenuated the expression of NPR-A as well as ANP-1–28-mediated increased levels of cGMP and suggest that the phosphorothioate oligonucleotide AS of NPR-A may be used as a tool to ablate the NPR-A and to define the physiological functions of NPR-A in vitro and maybe in vivo. The AS-induced knockdown of NPR-A was accompanied by the overexpression of NPR-C, which was time- and concentration-dependent. A significant increase of $\sim 70\%$ in NPR-C levels with a concomitant decrease of $\sim 70\%$ in NPR-A levels was observed at 5 $\mu$M and 48 h of treatment and suggests a reciprocal relationship between the two receptors. This notion is further supported by the results showing that the re-expression of NPR-A in AS-treated cells decreased the enhanced levels of NPR-C to control levels. In addition, the fact that overexpression of NPR-A in A10 VSMC resulted in the down-regulation of NPR-C strongly supports a cross-talk between NPR-A and NPR-C. Additional evidence for this interaction was provided by the results showing that silencing of NPR-A by using siRNA of NPR-A also resulted in the up-regulation of the expression of NPR-C. The enhanced expression of NPR-C by AS treatment was also reflected in enhanced inhibition of AC by C-ANP-4–23, a specific activator of NPR-C, resulting in the decreased levels of cAMP. Furthermore, the increased expression of NPR-C and NPR-C-mediated enhanced AC inhibition by AS treatment was specific for AS oligonucleotide and was not exhibited by sense oligomer. We also showed that the treatment of cells with AS did not affect the levels of Ang II receptor subtype AT1 and muscarinic receptor M2 subtype which like NPR-C is also coupled to AC through Gi protein and suggest that a cross-talk exists between NPR-A and NPR-C, the members of NPR family only, and not between NPR-A and other families of hormone receptors. Our results showing a cross-talk between the receptors of the same family are consistent with other reports showing that the blockade of AT1 receptor by losartan up-regulated the expression of AT2 receptor and unmasked AT2 receptor-mediated relaxation in response to Ang II in the aorta from hypertensive rats. Similarly, Sun et al. have demonstrated that the inhibition of the $\alpha_{1D}$-adrenergic receptor (AR) gene by RNA interference resulted in the overexpression of $\alpha_{2A}$-AR protein in the rat aortic VSMC.

Figure 6 Decreased levels of intracellular cGMP due to knockdown of NPR-A contributes to the enhanced expression of NPR-C and Giα protein through MAP kinase pathway. A10 VSMC were pre-incubated in the absence (control) or presence of 8Br-cGMP (0.5 mM) or PD 98059 (1 $\mu$M) for 1 h and thereafter with 5 $\mu$M NPR-A AS for 48 h. Cell lysates were prepared and subjected to western blotting using specific antibodies against NPR-C (A–D), Giα-2 (B–E), Giα-3 (C–F), or phospho-specific antibody against ERK1/2 (G). The proteins were quantified by densitometric scanning (lower panels). The results are expressed as a percentage of control taken as 100%. Values are means ± SE of three separate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. 

Cross-talk between NPR-A and NPR-C
The enhanced inhibition of AC by Ang II and ooxotremorine induced by AS treatment in the absence of enhanced expression of AT1 and M2 receptors may be attributed to the enhanced expression of Giα proteins that couple the AT1 and M2 receptors to the AC system. This notion is supported by our results showing that PT treatment attenuated AT1 and M2 receptor-mediated enhanced inhibition of AC in AS-treated cells. On the other hand, NPR-C-mediated enhanced inhibition of AC in AS-treated cells may be attributed to the up-regulation of both NPR-C and Giα protein because PT treatment also attenuated NPR-C-mediated inhibition of AC in AS-treated cells. In addition, the diminished stimulation of AC by isoproterenol, glucagon, and FSK by NPR-A AS treatment, resulting in decreased levels of cAMP, may not be attributed to Giα, because the levels of Giα were not altered by AS treatment, but may be due to the overexpression of Giα proteins. In this regard, the interaction between Giα and Gsα is well established. For example, the overexpression of Giα proteins has been shown to attenuate the stimulatory responses of hormones on AC activity, whereas the down-regulation of Giα proteins augmented the hormonal stimulation of AC activity. Furthermore, the results showing that AS treatment also decreased basal as well as FSK-stimulated AC activity suggest that knockdown of NPR-A by AS impaired the AC system, resulting in the decreased levels of cAMP.

The mechanism by which knockdown of NPR-A up-regulates the expression of NPR-C and Giα proteins appears to be attributed to the decreased levels of intracellular cGMP, because the treatment of AS-treated cells with 8Br-cGMP reversed the enhanced levels of NPR-C as well as of Giα proteins to control levels. This notion is supported by our earlier studies showing that the treatment of VSMC with 8-Br-cGMP as well as with nitric oxide, which stimulates soluble guanylyl cyclase and increases the levels of intracellular cGMP, down-regulated the expression of NPR-C and Giα proteins in VSMC. The signalling mechanism by which the decreased levels of intracellular cGMP due to NPR-A AS treatment up-regulate the expression of NPR-C and Giα proteins appears to involve MAP kinase pathway because AS treatment augmented the phosphorylation of ERK1/2 that was attenuated by 8Br-cGMP. Furthermore, the fact that the enhanced expression of NPR-C and Giα proteins evoked by NPR-A AS was attenuated by MEK inhibitor PD98059 further supports the role of MAP kinase in AS-induced up-regulation of NPR-C and Giα proteins.

The enhanced expression of Giα proteins and resultant decreased levels of cAMP have been shown in various models of hypertensive rats including spontaneously hypertensive rats, DOCA-salt hypertensive rats, 1-kidney-1-clip hypertensive rats, and L-NAME hypertensive rats and suggested the implication of enhanced levels of Giα proteins in the pathogenesis of hypertension. Furthermore, NPR-A knockout mice have also been shown to exhibit an increased blood pressure. Thus, taken together, it is possible that NPR-A knockout mice may also express the enhanced levels of Giα proteins and NPR-C, which by decreasing cAMP levels may contribute to the increased blood pressure in these knockout mice. However, this possibility needs to be investigated.

In conclusion, we provide evidence that knockdown of NPR-A up-regulates the expression of NPR-C as well as Giα proteins and suggests a cross-talk between the two receptors. It may be that the interplay of both the receptors NPR-A and NPR-C and not only one receptor subtype per se contributes to the regulation of physiological functions under pathophysiological conditions, including hypertension.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References


