Nitric oxide attenuates the expression of natriuretic peptide receptor C and associated adenyl cyclase signaling in aortic vascular smooth muscle cells: role of MAPK

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Arejian M, Li Y, Anand-Srivastava MB. Nitric oxide attenuates the expression of natriuretic peptide receptor C and associated adenyl cyclase signaling in aortic vascular smooth muscle cells: role of MAPK. Am J Physiol Heart Circ Physiol 296: H1859–H1867, 2009. First published February 27, 2009; doi:10.1152/ajpheart.01108.2008.—We have earlier shown that the treatment of A10 vascular smooth muscle cells with S-nitroso-N-acetyl-penicillamine (SNAP); nitric oxide donor (NO) for 24 h decreased the expression of natriuretic peptide receptor C (NPR-C) and adenyl cyclase signaling. The present study was undertaken to examine the implication of different signaling mechanisms in a NO-induced response. The treatment of A10 vascular smooth muscle cells with SNAP decreased the expression of NPR-C and Gα proteins in a time-dependent manner. The expression of Gα proteins was decreased at 6 h, whereas the expression of NPR-C was attenuated at 2 h. The NPR-C-mediated inhibition of adenyl cyclase was attenuated (∼50%) after 2 h of treatment and was completely abolished after 6 h of treatment. The decreased expression of NPR-C and NPR-C-mediated attenuation of adenyl cyclase after 2 h of treatment was reversed to control levels by PD-98059, a MEK inhibitor. SNAP also modulated the ERK1/2 phosphorylation in a time-dependent manner; an increase was observed up to 2 h, and, thereafter, the ERK1/2 phosphorylation was decreased. On the other hand, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and KT-5823 inhibitor of soluble guanylyl cyclase and protein kinase G, respectively, and Mn(III)tetrakis(4-benzoic acid)porphyrin, a scavenger of peroxynitrite, were unable to restore the SNAP-induced decreased expression of NPR-C protein and increased ERK1/2 phosphorylation to control levels. However, the decreased levels of phosphorylated ERK1/2 and Gα proteins were restored to control levels by 8-bromo-cAMP. These results indicate that a temporal relationship follows between a NO-induced decreased expression of NPR-C and Gα proteins. The decreased expression of NPR-C is mediated through cGMP-independent but MAPK-dependent pathway, whereas NO-induced decreased levels of cAMP may contribute to the decreased activation of MAPK and thereby decreased the expression of Gα proteins.

G proteins; mitogen-activated protein kinase

NATRIURETIC PEPTIDES (NPs) are a family of three peptide hormones termed atrial NP (ANP), brain NP, and C-type NP, all of which are produced in mammalian hearts, including human hearts (31). ANP regulates a variety of physiological parameters, including blood pressure (7), by interacting with receptors on plasma membrane either to generate second messengers such as cAMP (2, 3) and cGMP (20, 48) or to affect ion channels (7). Three types of NP receptors (NPRs) have been cloned; NPR-A (16) and NPR-B (15) subtypes are membrane-bound guanylyl cyclases (130-180 kDa) that mediate the biological actions of NP through a regulation of intracellular cGMP levels (7), whereas NPR-C (4, 17) is a disulfide-bridged homodimer of 67- and 77-kDa subunits with a broad range of ligands including ANP, brain NP, C-type NP, and des[Gln18, Ser19, Gly20, Leu21, Gly22]ANP4-23-amide C-ANP (C-ANP4-23), which does not show any affinity for NPR-A and NPR-B (51, 52). The 77-kDa subunit of NPR-C is involved in NP internalization and thus serves as a clearance receptor (51, 52), whereas the 66-kDa subunit is coupled to adenyl cyclase inhibition through the inhibitory guanine nucleotide regulatory protein Gα (4, 5) or to the activation of phospholipase C (21).

The adenyl cyclase/cAMP signal transduction system is implicated in the regulation of various physiological functions including vascular tone and reactivity, cardiac functions, platelet functions, etc. The adenyl cyclase system is composed of three components: receptor, catalytic subunit, and guanine nucleotide regulatory protein (G protein). The stimulatory and inhibitory responses of the hormones on adenyl cyclase are mediated by stimulatory (Gs) and inhibitory (Gi) proteins, respectively (19). G proteins exist as heterodimers of α-, β-, and γ-subunits, and the specificity of G proteins is attributed to α-subunit. Molecular cloning has revealed four different forms of Gα resulting from the differential splicing of one gene (13) and three distinct forms of Gα (Gα-1, Gα-2, and Gα-3) encoded by three different genes (22). All three isoforms of Gα are implicated in the regulation of adenyl cyclase inhibition (22). The NPR-C and associated Gα protein-adenyl cyclase signaling have been reported to be regulated by various vasoactive peptides such as angiotensin II, ANP, endothelin, and arginine-vasopressin (9–12, 37).

Nitric oxide (NO) is a diffusible universal messenger that acts as an important biological signaling molecule in diverse physiological processes, including vasorelaxation (29). Both NO and NP function as vasodilators by the activation of the guanylate cyclase/cGMP pathway (34). NO stimulates soluble guanylyl cyclase (sGC), whereas NP activates the membrane-bound particulate guanylyl cyclase of NPR-A/NPR-B and increases cGMP levels (34). NO has also been shown to act by cGMP-independent mechanisms through the activation of MAPKs (29, 46). We have recently shown that a longer treatment of vascular smooth muscle cells (VSMCs) with S-nitroso-N-acetyl-penicillamine (SNAP) for 24 h decreased the levels of NPR-C and attenuated the NPR-C-mediated inhibition of adenyl cyclase activity; however, the underlying mechanism responsible for this attenuation is not known. The
present studies were therefore undertaken to investigate whether the short-term treatment of VSMCs with SNAP also resulted in a decreased expression of NPR-C and an associated adenyl cyclase signaling and to examine the underlying mechanism responsible for NO-induced effects.

MATERIALS AND METHODS

α-[32P]ATP was purchased from GE HealthCare BioSciences (Montreal, QC, Canada). Des[Gln18, Ser19, Gly20, Leu21, Gly22]ANP4-23-amide C-ANP (C-ANP4-23) was from Bachem. Mn(III)tetraakis(4-benzoic acid)porphyrin (MnTBPAP) and KT-5823 were purchased from Cedarlane (Hornby, ON, Canada). PD-98059 was from Sigma-Aldrich (St. Louis, MO). All antibodies and Western blot analysis reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma-Aldrich.

Cell culture and incubation. Rat aortic VSMCs were cultured as described previously (1). A-10 cell line (A10 VSMCs) from rat embryonic thoracic aorta was obtained from American Type Culture Collection (Rockville, MD). The cells were plated in 75-cm² flasks or in 100-mm plates and were incubated at 37°C in 95% room air-5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) (with glucose, 1-glutamine, and sodium bicarbonate) containing 1% antibiotics and 10% heat-inactivated fetal bovine serum (FBS). VSMCs and A10 cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA (VSMCs were used between passages 4 and 10). Confluent cells were starved by incubation for 3 or 24 h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. The cells were then incubated in the absence or presence of 100 μM SNAP for different time periods (2–24 h). To examine the effect of various inhibitors, the cells were incubated in the absence or presence of PD-98059 (10 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 20 μM), KT-5823 (1 μM), or MnTBPAP (20 μM) 30 min before the treatment with SNAP (100 μM) for 2 h. After incubation for different time periods at 37°C, the cells were harvested by chemical lysis or were homogenized in a Dounce homogenizer (Wheaton, Millville, NJ). The homogenate was collected by centrifugation at 4°C for 10 min at 2,800 rpm and resuspended in Tris-EDTA buffer and was used for adenyl cyclase assay. On the other hand, cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C and were used for immunoblotting studies. Cell viability was checked with the trypan blue exclusion technique. All the protocols used in the present study were approved by the Comité de Deontologie de l’expérimentation sur les Animaux (Canada).

Adenyl cyclase activity determination. Adenyl cyclase activity was determined by measuring [32P]cAMP formation from α-[32P]ATP as described previously (4).

Western blot analysis. Western blot analysis of G1 proteins, NPR-C, and MAPK was performed as described previously (30). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Bio-Rad) with semidy transblot apparatus (Bio-Rad). The proteins on the membranes were stained with Ponceau S to confirm that an equivalent amount of proteins were loaded into each well. The membranes were then blocked for 1 h at room temperature in PBS containing 5% nonfat dry milk and 0.2% Tween 20 and were then incubated with antibodies directed against NPR-C (N-20), Gα-2 (AS7), Gα-3 (EC2), phosphorylated ERK (E-4) (against phosphorylated Tyr204 of ERK1/2), total ERK (C-14), and dynein (74-1) (used as control for protein loading) overnight at 4°C. The antibody-antigen complexes were detected by incubating the membranes with horseradish peroxidase-conjugated antibodies for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence Western blot analysis detection reagents from Santa Cruz. The autoradiograms were quantified by densitometric scanning using an enhanced laser densitometer (LKB Ultrason XL, Pharmacia) and the gel scan evaluation software (version 2.1) from Pharmacia. The scanning was one dimensional and scanned the entire area of the protein bands in autoradiograms.

RESULTS

Effect of SNAP treatment on NPR-C protein levels in A10 VSMCs. We have recently shown that exposure of A10 VSMCs with SNAP for a longer period of time (24 h) decreased the expression of NPR-C (10). To investigate whether the treatment of cells with SNAP for a shorter period of time also resulted in a decreased expression of NPR-C protein, we treated A10 VSMCs with 100 μM SNAP for different time periods and the levels of NPR-C protein were determined by immunoblotting using a specific antibody directed against the NPR-C protein. The results shown in Fig. 1 indicate that SNAP significantly (~40%) decreased the levels of NPR-C protein at 2 h of treatment and remained decreased until 24 h of treatment (Fig. 1B). However, treatment of A10 VSMCs for shorter periods of time (0.5–1 h) did not alter the expression of NPR-C (Fig. 1A).

Effect of SNAP treatment on NPR-C-mediated inhibition of adenyl cyclase. To investigate whether the decreased levels of NPR-C by SNAP treatment were reflected in NPR-C-mediated inhibition of adenyl cyclase, the effect of SNAP treatment was examined on C-ANP4-23-mediated inhibition of adenyl cyclase in A10 VSMCs. The results shown in Fig. 2 indicate that C-ANP4-23, which interacts specifically with NPR-C, inhibited adenyl cyclase activity in a concentration-dependent manner in control cells, with an apparent inhibitory constant (Ki) of about 5 nM. The maximal inhibition observed was about 45%. However, this inhibition was attenuated by about 50% (45 vs. 20%) in cells treated with SNAP for 2 h (short term), whereas the longer treatment of cells (6 h) with SNAP completely abolished the C-ANP4-23-mediated inhibition of adenyl cyclase that persisted unto 24 h (data not shown).

Effect of SNAP treatment on G1 protein levels in A10 VSMCs. A partial downregulation of NPR-C and a complete attenuation of NPR-C-mediated inhibition of adenyl cyclase by C-ANP4-23 for 6 h may suggest the involvement of postreceptor components in the desensitized response. To investigate this possibility, the levels of Gα1 proteins that couple NPR-C to adenyl cyclase were determined by the immunoblotting technique using specific antibodies against Gα-2 and Gα-3 in control and SNAP-treated cells. As reported previously (10), AS7 antibodies that react with both Gα-1 and Gα-2 and EC2 antibodies against Gα-3 recognized a single protein band of 40 (Gα-2) and 41 kDa (Gα-3) in control and SNAP-treated cells (Fig. 3). The levels of Gα-2 (Fig. 3A) and Gα-3 (Fig. 3B) were not altered at 2 h of treatment with SNAP; however, these started decreasing at 6 h of treatment (~20%), and a decrease of about 30–35% was observed at 24 h as determined by densitometric scanning. These results suggest that C-ANP4-23-mediated inhibition of adenyl cyclase by about 50% as observed after 2 h of SNAP treatment may not be attributed to the decrease in the levels of Gα proteins and may be due to the reduction in NPR-C. But when the levels of Gα proteins were also inhibited (20–40%) after 6 h or a longer period of treatment with...
SNAP, a complete attenuation of C-ANP4-23-mediated inhibition of adenylyl cyclase was observed.

Effect of SNAP treatment on \( G_i \) functions. To investigate whether a SNAP-induced decreased expression of \( G_\alpha \) proteins was also reflected in \( G_i \) functions, the effect of low concentrations of GTP\( \gamma \)S on forskolin (FSK)-stimulated adenylyl cyclase activity (receptor-independent \( G_i \) functions) was examined in control and SNAP-treated cells. The results shown in Fig. 4 indicate that GTP\( \gamma \)S inhibited FSK-stimulated adenylyl cyclase activity in a concentration-dependent manner in control cells, and the maximal inhibition of about 40% was observed at \( 10^{-9} \) M GTP\( \gamma \)S. However, the treatment of cells with SNAP for 6 h completely abolished this inhibition. On the other hand, the treatment of cells with SNAP for 2 h did not affect the extent of GTP\( \gamma \)S-mediated inhibition of FSK-stimulated adenylyl cyclase activity (data not shown). However, the basal adenylyl cyclase activity without GTP\( \gamma \)S was significantly attenuated (control cells, 78.5 \( \pm \) 2.1; and SNAP-treated cells, 39.1 \( \pm \) 3.2 pmol cAMP\( \cdot \)mg protein\( ^{-1} \)\( \cdot \)10 min\(^{-1} \)).

Effect of ODQ, KT-5823, and MnTBAP on SNAP-induced decreased expression of NPR-C. Since NO has been shown to act via cGMP-dependent and -independent pathways (29), it was of interest to examine whether NO-induced decreased expression of NPR-C protein is mediated through cGMP pathway or involves some other mechanisms. To test this, we examined the effect of ODQ, an inhibitor of sGC, on the expression of NPR-C protein in control and SNAP-treated A10 VSMCs. The results shown in Fig. 5A indicate that ODQ was unable to reverse the SNAP-induced decreased levels of NPR-C to control levels; however, ODQ inhibited SNAP-induced increased levels of cGMP (data not shown).

To further confirm our finding, we tested the effect of KT-5823, a protein kinase G inhibitor, on the SNAP-induced decreased expression of NPR-C protein in A10 VSMCs. The result shown in Fig. 5B indicates that KT-5823, like ODQ, was also unable to restore the SNAP-induced decreased level of NPR-C. These data strongly suggest that NO-induced decreased expression of NPR-C protein occurs through a cGMP-independent mechanism.

NO has been reported to form peroxynitrite by its interaction with superoxide anion in VSMCs (31). To investigate whether NO-induced decreased level of NPR-C protein was attributed...
to the formation of peroxynitrite, we investigated the effect of MnTBAP, a scavenger of peroxynitrite and a superoxide dismutase mimetic agent, on the SNAP-induced decreased level of NPR-C protein in A10 VSMCs. The results shown in Fig. 5C reveal that MnTBAP did not restore the SNAP-induced decreased expression of NPR-C protein toward control levels, suggesting that peroxynitrite was not responsible for the NO-mediated decreased expression of NPR-C protein.

**Effect of MEK inhibitor PD-98059 on SNAP-induced decreased expression of NPR-C.** NO has been shown to regulate MAPK activity (24, 28). To investigate whether SNAP-induced decreased expression of NPR-C is mediated through MAPK activation in VSMCs, we examined the effect of PD-98059, a MEK inhibitor, on NPR-C levels in A10 VSMCs treated with SNAP for 2 h. The results shown in Fig. 6A illustrate that the SNAP-induced decreased levels of NPR-C were restored to control levels by PD-98059, suggesting the implication of MAPK in SNAP-induced decreased levels of NPR-C. To further investigate whether the PD-98059-induced restoration of the decreased expression of NPR-C evoked by SNAP was also reflected in the restoration of the NPR-C-mediated inhibition of adenylyl cyclase, the effect of PD-98059 was examined on the NPR-C-mediated inhibition of adenylyl cyclase activity in SNAP-treated cells. As shown in Fig. 6B, PD-98059 treatment also restored the C-ANP4-23-induced decreased inhibition of adenylyl cyclase in SNAP-treated cells to control levels. However, PD-98059 treatment alone did not alter the C-ANP4-23-mediated inhibition of adenylyl cyclase (data not shown).

**Effect of SNAP treatment on ERK1/2 phosphorylation.** To further investigate whether SNAP could also modulate the phosphorylation of ERK1/2 at 2 h, we examined the effect of SNAP on ERK1/2 phosphorylation at different time periods. The results shown in Fig. 7 illustrate that control cells exhibit basal level of phosphorylation of ERK1/2 which was enhanced by about 60% by SNAP at 2 h and decreased thereafter, and at 6 h, the ERK1/2 phosphorylation was decreased by about 30% compared with control levels. In addition, the enhanced phos-
of protein bands shown in Fig. 8 indicate that these inhibitors were unable to reverse the SNAP-induced decreased phosphorylation of ERK1/2 in these cells.

Effect of 8-bromo-cAMP on SNAP-induced decreased levels of Gα proteins and ERK1/2 phosphorylation. cAMP has been reported to modulate MAPK activity (36, 45). Since SNAP treatment decreases the basal activity of adenylyl cyclase and results in decreased levels of cAMP, it may be possible that the decreased levels of cAMP induced by SNAP may be responsible for SNAP-evoked decreased ERK1/2 phosphorylation at 6 h and thereby decreased levels of Gα proteins. To investigate this possibility, the effect of 8-bromo-cAMP (8-Br-cAMP) on the levels of Gα proteins and ERK1/2 phosphorylation was examined in A10 VSMCs treated with SNAP for 6 h. The results shown in Fig. 9 indicate that SNAP-induced decreased ERK1/2 phosphorylation (Fig. 9A) and decreased levels of Gα-2 (Fig. 9B) and Gα-3 (Fig. 9C) were restored to control levels by 8-Br-cAMP, suggesting the role of cAMP in SNAP-induced decreased levels of phosphorylated ERK1/2 and Gα proteins.

**DISCUSSION**

The present study demonstrates that pretreatment of A10 VSMCs by SNAP for 2 h results in the downregulation of NPR-C without altering the levels of Gα proteins. This down-regulation of NPR-C was reflected in the attenuation of NPR-C-mediated inhibition of adenylyl cyclase. However, a longer treatment of A10 VSMCs with SNAP for 6 h that downregulates the NPR-C completely abolishes the NPR-C-mediated inhibition of adenylyl cyclase. These results are in accordance with our previous studies which showed that a longer treatment of A10 VSMCs with SNAP (24 h) abolished the NPR-C-mediated inhibition of adenylyl cyclase (10); however, these studies did not examine the short-term effect of SNAP on NPR-C expression. A complete attenuation of NPR-C-mediated inhibition of adenylyl cyclase with a partial downregulation of NPR-C (~30%) after 6 h of SNAP treatment is in agreement with other studies showing that the inhibition of NPR-C expression by about 50% by NPR-C antisense (39) or by about 35% by pretreatment with C-ANP4-23 (9) resulted in a complete attenuation of the NPR-C-mediated inhibition of adenylyl cyclase in A10 VSMCs and suggests that a post-receptor modification, such as an alteration in the levels of Gα proteins that couple NPR-C to adenylyl cyclase, may also be responsible for the observed desensitization of adenylyl cyclase inhibition. In this regard, a relationship between the levels of Gα proteins and functions has been reported by several investigators (6, 8, 9, 38).

In the present study, we show that SNAP-induced decreased levels of Gα proteins after 6 h of treatment may partially contribute to the attenuation of NPR-C-mediated inhibition of adenylyl cyclase. However, the fact that a shorter period of treatment (2 h) that attenuated NPR-C-mediated inhibition by about 50% without altering the levels of Gα proteins suggests that Gα proteins may not be implicated in the desensitization of NPR-C-mediated inhibition of adenylyl cyclase at early period of SNAP treatment. However, when the levels of Gα proteins were phosphorylation of ERK1/2 observed at 2 h was restored to control levels by PD-98059.

Effect of ODQ, KT-5823, and MnTBAP on SNAP-induced decreased phosphorylation of ERK1/2. To investigate the role of cGMP and peroxynitrite on the SNAP-induced decreased phosphorylation of ERK1/2 (6 h), the effect of ODQ, KT-5823, and MnTBAP on the phosphorylation of ERK1/2 was examined at 6 h in control and SNAP-treated VSMCs. The results shown in Fig. 8 indicate that these inhibitors were unable to reverse the SNAP-induced decreased phosphorylation of ERK1/2 in these cells.

Fig. 5. Effect of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), KT-5823, and MnIII[Tetrakis(4-benzoic acid)]porphyrin (MnTBAP) on SNAP-induced decreased expression of NPR-C protein in A10 VSMCs. A–C, *top: A10 VSMCs were incubated in the absence (control) or presence of 20 μM ODQ (A), 1 μM KT-5823 (B), or 20 μM MnTBAP (C) 30 min before the treatment with SNAP (100 μM) for 2 h. Membrane were prepared and 50 μg of membrane proteins from control, and treated cells were used for Western blot analysis for NPR-C protein expression as described in MATERIALS AND METHODS. A–C, bottom: quantification of protein bands shown in top by densitometric scanning. The results are expressed as percentages of control taken as 100%. Values are means ± SE of 4 separate experiments. ***P < 0.001 vs. control, NS, not significant.
also inhibited upon a longer exposure (6 h), a complete attenuation of NPR-C-mediated inhibition of adenylyl cyclase was observed. These data strongly suggest that a complete attenuation of NPR-C-mediated inhibition of adenylyl cyclase after 6 h of SNAP treatment may not be solely attributed to the decreased levels of $G_{i\alpha}$ proteins that couple NPR-C to adenylyl cyclase or to the decreased expression NPR-C but most likely reflects a cumulative effect of both the components.

We also showed that NO-induced decreased expression of NPR-C protein was not blocked by ODQ, an inhibitor of sGC, and KT-5823, an inhibitor of protein kinase G, and suggest that NO-induced decreased expression of NPR-C protein was not mediated through a cGMP-dependent mechanism and may involve some other mechanisms. In this regard, increasing evidence indicates that NO regulates a variety of physiological functions in a cGMP-independent mechanism, including apoptosis and growth inhibition in vascular and endothelial cells (49). In addition, peroxynitrite, which is formed by the interaction of NO and superoxide anion (50), did not appear to be implicated in NO-induced decreased expression of NPR-C.

Fig. 6. Effect of PD-98059 (PD) on SNAP-induced decreased NPR-C expression and C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase activity in A10 VSMCs. A, top: A10 VSMCs were incubated in the absence (control) or presence of 10 μM PD-98059 30 min before the treatment with SNAP (100 μM) for 2 h. Membranes were prepared and 50 μg of membrane proteins from control, and treated cells were used for Western blot analysis for NPR-C protein expression as described in MATERIALS AND METHODS. A, bottom: quantification of protein bands shown in top by densitometry scanning. The results are expressed as percentages of control taken as 100%. Values are means ± SE of 4 separate experiments. B: adenylyl cyclase activity was determined in the membrane in the absence or presence of 10 μM GTP$_\gamma$S alone or in combination with various concentrations of C-ANP$_{4-23}$. Basal enzyme activities in the presence of 10 μM GTP$_\gamma$S in control, SNAP-treated cells, and PD + SNAP-treated cells were 354.5 ± 11.4 and 324.4 ± 12.3 and 369.6 ± 6.3 pmol cAMP·mg protein$^{-1}$·10 min$^{-1}$. Values are means ± SE of 6 separate experiments performed in triplicates. *$P < 0.05$ and **$P < 0.01$ vs. control.

Fig. 7. Time course of SNAP treatment on phosphorylation (p) of ERK1/2 and effect of PD-98059 on SNAP-induced increased pERK1/2 in A10 VSMCs. A10 VSMCs were incubated in the absence (control) or presence of 100 μM SNAP for different time periods or were incubated in the absence (control) or presence of 10 μM PD-98059 for 30 min before the treatment with SNAP (100 μM) for 2 h. The cell lysates (30 μg) from control and treated cells were used for Western blot analysis as described in MATERIALS AND METHODS. The results are expressed as percentages of control taken as 100%. Values are means ± SE of 4 separate experiments. **$P < 0.01$ vs. control.
because MnTBAP, a scavenger of peroxynitrite and superoxide dismutase mimetic agent, did not restore the expression of NPR-C toward control levels. On the other hand, SNAP-induced decreased expression of NPR-C and NPR-C mediated decreased inhibition of adenylyl cyclase after 2 h of treatment that was restored to control levels by PD-98059, a MEK inhibitor, suggests the implication of MAPK in SNAP-induced decreased expression and associated adenylyl cyclase signaling. This was further supported by our results showing that SNAP increased the ERK1/2 phosphorylation after 2 h of treatment, which was restored to control levels by PD-98059. Our results are in accordance with the studies of other investigators who showed that SNAP at 100–300 μM stimulated the ERK1/2 phosphorylation in various cell types including rabbit aortic endothelial cells (28, 36). In addition, sodium nitroprusside, another donor of NO, was also shown to stimulate ERK1/2 phosphorylation in rat cardiomyocytes (24). However, our studies are in contrast with the studies of Palen et al. (40) who have shown that treatment of aortic VSMCs with SNAP and sodium nitroprusside for 1–30 min decreased the ERK1/2 phosphorylation. These discrepancies may be due to the difference in the time of

**Fig. 8.** Effect of ODQ KT-5823 and MnTBAP on SNAP-induced decreased pERK1/2 in A10 VSMCs. A and B, top: A10 VSMCs were incubated in the absence (control) or presence of 20 μM ODQ, or 1 μM KT-5823 (A) or 20 μM MnTBAP (B) 30 min before the treatment with SNAP (100 μM) for 2 h. The cell lysates (30 μg) from control and treated cells were used for Western blot analysis as described in MATERIALS AND METHODS. A and B, bottom: quantification of protein bands as performed by densitometric scanning. The results are expressed as percentages of control taken as 100%. Values are means ± SE of 4 separate experiments. ***P < 0.001.

**Fig. 9.** Effect of 8-bromo-cAMP on SNAP-induced decreased pERK1/2 and decreased expression of G<sub>x</sub>-2 and G<sub>x</sub>-3 protein in A10 VSMCs. A–C, top: A10 VSMCs were incubated in the absence (control) or presence of 500 μM 8-bromo-cAMP 2 h before the treatment with SNAP (100 μM) for 6 h. Cell lysates (30 μg) from control and treated cells were used for Western blot analysis as described in MATERIALS AND METHODS. A–C, bottom: quantification of protein bands as performed by densitometric scanning. The results are expressed as percentages of control taken as 100%. Values are means ± SE of 4 separate experiments. *P < 0.05 vs. control.
treatment (2 h vs. 30 min). Taken together, we showed that SNAP-induced decreased expression of NPR-C and associated adenylyl cyclase signaling is cGMP independent and is mediated through the MAPK pathway.

We also showed a correlation between SNAP-induced decreased phosphorylation of ERK1/2 and \( \mathrm{G}_{\alpha} \) protein levels. The longer treatment of A10 VSMCs with SNAP for 6 h or more decreased ERK1/2 phosphorylation as well as the expression of \( \mathrm{G}_{\alpha}\alpha \)-2 and \( \mathrm{G}_{\alpha}\alpha \)-3 proteins. In this regard, NO-induced decreased activation of ERK1/2 has also been reported in rat VSMCs, mouse macrophage-like cell line, and rat alveolar macrophages (23, 36, 40). MAPK is implicated in many cellular functions such as proliferation, differentiation, migration, and cell death (27, 42). We recently showed the involvement of MAPK in angiotensin II-induced increased expression of \( \mathrm{G}_{\alpha}\alpha \)-2 and \( \mathrm{G}_{\alpha}\alpha \)-3 proteins in A10 VSMCs (18). Taken together, it may be possible that the SNAP-induced decreased activity of MAPK in these cells, as demonstrated by the reduced ERK1/2 phosphorylation, may be responsible for the decreased expression of \( \mathrm{G}_{\alpha}\alpha \)-2 and \( \mathrm{G}_{\alpha}\alpha \)-3 proteins. It should also be noted that the SNAP-induced decreased expression of \( \mathrm{G}_{\alpha} \) proteins as well as the decreased MAPK activation may not be attributed to apoptosis, because cell viability checked by the trypan blue exclusion technique indicated that >90–95% cells were viable. The mechanism by which SNAP decreased MAPK activity may not involve cGMP and peroxynitrite because ODQ, KT-5823, and MnTBP were unable to restore the SNAP-induced decreased phosphorylation of ERK1/2. However, the SNAP-induced decreased levels of cAMP may be responsible for the decreased ERK1/2 phosphorylation and thereby the decreased levels of \( \mathrm{G}_{\alpha}\alpha \)-2 and \( \mathrm{G}_{\alpha}\alpha \)-3 proteins. This notion is further supported by our results showing that the treatment of cells with 8-Br-cAMP restored the SNAP-induced decreased phosphorylation of ERK1/2 and the decreased levels of \( \mathrm{G}_{\alpha}\alpha \)-2 and \( \mathrm{G}_{\alpha}\alpha \)-3 protein observed at 6 h of SNAP treatment to control levels. However, the increased ERK1/2 phosphorylation by SNAP at 2 h may not be due to cAMP, because the levels of cAMP were decreased and not increased by SNAP treatment. In this regard, a chronic exposure of adipocytes with adenosine agonist N6-phenylisopropyladenosine that interacts with inhibitory adenosine receptor type 1 and inhibits adenylyl cyclase activity and cAMP levels has also been reported to decrease the levels of G\(_{\alpha}\) proteins (41). In addition, isoprenaline that stimulates adenylyl cyclase activity and cAMP levels has been shown to augment the levels of G\(_{\alpha}\) proteins (44).

The NO-induced decreased expression of \( \mathrm{G}_{\alpha} \) proteins and NPR-C may be a potential mechanism for some of the physiological effects. For instance, the role of \( \mathrm{G}_{\alpha} \) proteins in the regulation of proliferation and blood pressure has been reported (6, 35). We have earlier shown the implication of the enhanced expression of \( \mathrm{G}_{\alpha}\alpha \) protein in the pathogenesis of hypertension (32), as well as in hyperproliferation of VSMCs from spontaneously hypertensive rats compared with Wistar-Kyoto control rats (33). NO has also been reported to inhibit VSMC proliferation (44), as well as the development of pulmonary hypertension (26). Taken together, it may be suggested that NO-induced decreased levels of \( \mathrm{G}_{\alpha} \) proteins may be one of the contributing factors responsible for the antiproliferative action of NO and for the attenuation of blood pressure. In addition, it may be possible that NO-induced decreased expression of NPR-C may upregulate the expression of guanylyl cyclase receptor NPR-A and thereby increase cGMP levels that may result in the enhanced vasorelaxation and thereby reduction in the blood pressure. In this regard, a cross talk between NPR-C and NPR-A has been shown (25). Our earlier studies also showed that the knocking down of NPR-C by NPR-C antisense resulted in enhanced responsiveness of ANP to stimulate cGMP levels in A10 VSMCs (39). Furthermore, the NO-induced decreased expression of NPR-C that resulted in the attenuation of C-ANP\(_{23}\)-mediated inhibition of adenylyl cyclase and thereby increased the levels of cAMP may also contribute to the enhanced vasorelaxation and reduction in blood pressure.

In conclusion, we have shown that short-term exposure of VSMCs to SNAP decreased the expression of NPR-C and NPR-C-mediated adenylyl cyclase signaling through the activation of MAPK. However, the SNAP-induced decreased levels of NPR-C and \( \mathrm{G}_{\alpha} \) proteins at 6 h completely abolished the NPR-C-mediated adenylyl cyclase inhibition. It may be thus suggested that the NO-induced downregulation of NPR-C and \( \mathrm{G}_{\alpha} \) protein that results in the attenuation of adenylyl cyclase inhibition and thereby increases the levels of cAMP may be an additional mechanism responsible for the vasorelaxant effect of NO and the regulation of blood pressure.

GRANTS

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REFERENCES

NITRIC OXIDE MODULATES NPR-C PROTEIN EXPRESSION


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