Protein kinase C activation down-regulates natriuretic peptide receptor C expression via transcriptional and post-translational pathways

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Received 2 October 1997; revised version received 30 October 1997

Abstract Natriuretic peptide receptor C (NPR-C) mRNA expression and ANP-binding activity via NPR-C are significantly down-regulated in HeLa cells with phorbol myristate acetate (PMA) treatment. Stabilization of the NPR-C mRNA by PMA indicated that down-regulation of its mRNA was mediated through negative transcriptional regulation. Despite the significant loss of the mRNA, reduction of NPR-C-specific ANP-binding activity after PMA exposure (4 h) was accompanied by a slight decrease in total NPR-C protein (with a 5% loss) and was also produced in the presence of actinomycin D or cycloheximide. The inhibitory effect of a long PMA exposure (18 h) paralleled with a decrease in total NPR-C protein is suggested to be dependent on reduction of de novo NPR-C synthesis. PMA-induced transcriptional and post-translational down-regulation of NPR-C was effectively reversible in the presence of the protein kinase C inhibitor GF109203X. These findings demonstrate that protein kinase C activation down-regulated NPR-C expression through transcriptional and post-translational pathways and that immediate functional receptor loss was mediated via a post-translational mechanism, such as enhanced receptor internalization.

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Key words: Natriuretic peptide; Natriuretic peptide clearance receptor; Phorbol myristate acetate; Down-regulation; Post-translational pathway

1. Introduction

Natriuretic peptides (NPs) are known to play important roles in cardiovascular homeostasis. Three isoforms, termed atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) [1,2], constitute the natriuretic peptide family. ANP and BNP are considered to be responsible for systemic blood pressure control and body fluid homeostasis [3,4]. In contrast, CNP has recently been demonstrated to be involved in the suppression of vascular smooth muscle cells proliferation and in the phenotypic development of osteoblasts [5]. These biological functions of NPs are mediated via intracellular production of guanosine 3′,5′-cyclic monophosphate (cGMP) through guanylyl cyclase (GC)-coupled receptors, termed GC-A and GC-B. Natriuretic peptide receptor C (NPR-C) putatively has a very short intracytoplasmic domain which lacks guanylyl cyclase activity [6–8]. While this receptor supposedly regulates the biological effects of NPs by elimination from the circulation, NPR-C has also been reported to control directly adenylyl cyclase activity via a Gi protein, phospholipid hydrolysis, thymidine kinase activity and mitogen-activated protein kinase activity in the absence of intracellular cGMP production [9–13].

Several significant studies have been done on characterizing NPR-C expression in vitro and in vivo. Increases in intracellular cAMP and cGMP concentrations have been shown to inhibit NPR-C gene expression [14,15], whereas down-regulation of the receptor in the lungs of rats adapted to hypoxia, in the kidneys of rats with renal failure and streptozotocin-induced diabetes mellitus, and in the aorta of hypertensive rats has been reported [16–19]. The pathophysiological loss of the NPR-C protein is suggested to reduce the elimination rate of NPs. Although NPR-C has been demonstrated to localize in the tracheal and glomerular epithelium [20–22] by immunohistological studies, no information is yet available on the mechanisms underlying the down-regulation of NPR-C expression.

Here, we show that protein kinase C activation significantly and independently down-regulates NPR-C mRNA expression and NPR-C-dependent ANP-binding activity in human epithelial-like cells via transcriptional and post-translational regulation. We recently reported highly specific antisera that recognize the NPR-C cytoplasmic domain [22], and the availability of these antisera enabled us to investigate NPR-C expression at the total protein level.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan). [α-^{32}P]dCTP, [^{125}I]human ANP and Hybond-N$^+$ nylon filter were from Amersham. Human ANP was purchased from The Peptide Institute (Osaka, Japan). The ANP analog, des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANP-(4–23)-NH_2 (C-ANF(4–23)), and bovine serum albumin (fraction V) were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FCS) were obtained from Gibco Laboratories. GF109203X was purchased from Calbiochem (USA).

2.2. Cell cultures and transient cell transfection

HeLa cells, purchased from Dainippon Pharmaceutical Co. (Osaka, Japan), were cultured in DMEM supplemented with 10% FCS and passaged in a controlled atmosphere of 5% CO_2/95% air at 37°C. HeLa cells were washed twice with PBS and incubated in DMEM containing 0.1% bovine serum albumin with phorbol myristate acetate (PMA, 20 ng/ml) for 2 h, 4 h or 18 h.

2.3. Binding assay of NPR-C

HeLa cells in 24-well dishes were washed twice with 1 ml of PBS. Binding of 0.5 nM [^{125}I]human ANP was allowed to proceed in DMEM containing 0.1% bovine serum albumin for 30 min at 4°C to equilibrium in the presence or absence of unlabeled C-ANF(4–23) (1 μM) ($n=4$). After 30 min, cells were washed twice with PBS and

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Abbreviations: ANP, atrial natriuretic peptide; NPR-C, natriuretic peptide receptor C; PKC, protein kinase C; PMA, phorbol myristate acetate; GAPDH, glyceraldehyde phosphate dehydrogenase

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PII S0014-5793(97)01406-3

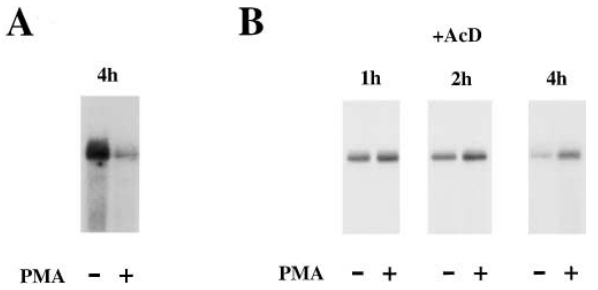

Fig. 1. RNA analysis by Northern blot hybridization. Hybridization was carried out with a fragment of human NPR-C cDNA as a probe as described in Section 2. A: HeLa cells were incubated for 4 h in the presence or absence of PMA. 5 μg of poly(A)$^+$ RNA was subjected to Northern blotting analysis. The filter was subjected to autoradiography for 16 h. B: HeLa cells were incubated for 30 min with actinomycin D (5 μg/ml) prior to treatment with PMA. 5 μg of poly(A)$^+$ RNA of the indicated time was prepared for Northern blot analysis. The filter was subjected to autoradiography for 72 h.

solubilized with 500 μl of 0.5 N NaOH. Aliquots were assayed for radioactivity using a Cobra γ-counter (Packard).

2.4. Western blot (immunoblot) analysis

The antiserum against the NPR-C protein used was obtained by the following procedure as reported previously [22]. Mitogen-activated protein (MAP)-peptide complex containing the sequence (NH_2-RKKYRITIERRNHQEESNIGKHRELREDSIRSHFSVA-COOH), derived from the cytoplasmic domain of rat NPR-C protein, was synthesized by the 9-fluorenylmethyloxycarbonyl synthesis strategy. Polyclonal antibody toward the peptide was obtained by injecting rabbits with the peptide in Freund's complete adjuvant. HeLa cells were homogenized in 3 ml of buffer A (10 mM Tris (pH 7.5), 5 mM EDTA, 5 μM leupeptin, 2 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). After centrifugation for 30 min at 5000×g at 4°C, the resulting supernatants were centrifuged for 30 min at 40 000×g at 4°C. 20 μg of the precipitates re-suspended in 200 μl of buffer A were subjected to SDS-PAGE (10% polyacrylamide). The proteins were electrophoretically transferred to an Immobilon P filter (Millipore) for immunodetection. The blots were blocked by soaking in 5% non-fat dried milk in PBS and incubated with anti-NPR-C antiserum (diluted 1:1000) for 18 h at 4°C. Signals were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and the enhanced chemiluminescence system (Amersham).

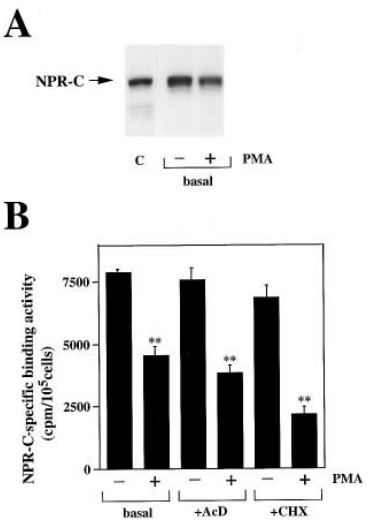

Fig. 2. Western blot analysis and ANP-binding assay using actinomycin D and cycloheximide. A: HeLa cells were incubated for 4 h in the presence or absence of PMA. Western blot analysis was carried out as described in Section 2. C = positive control, 45 μg of membrane protein from rat lung. B: After HeLa cells were incubated for 30 min with actinomycin D (5 μg/ml) or cycloheximide (5 μg/ml), cells were incubated for 4 h in the presence or absence of PMA. $^{**}P < 0.01$ compared with basal.

2.5. RNA analysis by Northern blot hybridization

Total RNA was isolated from HeLa cells using Isogen (Nippon gene, Toyama, Japan), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose column chromatography using mRNA separator kit (Clontech). 5 μg of poly(A)$^+$ RNA was fractionated in a 1% agarose gel containing 0.66 M formaldehyde and 0.02 M morpholinopropanesulfonic acid (pH 7.0). Fractionated RNAs were transferred onto a nylon filter by capillary blotting and then crosslinked by UV irradiation. A ^{32}P-labeled cDNA fragment encoding human NPR-C or glyceraldehyde phosphate dehydrogenase (GAPDH) was used for Northern blot hybridization as probes. The filter was washed three times in 1×SSC, 0.1% SDS at 55°C for 30 min each and subjected to autoradiography for the indicated times.

2.6. Statistical analysis

All values are expressed as mean ± S.D. Statistical significance was determined by the unpaired Student's t-test.

3. Results

NPR-C mRNA expression was significantly down-regulated in HeLa cells treated with PMA (Fig. 1A). The steady-state mRNA level as determined above can be affected by the rate of mRNA degradation as well as by that of gene transcription. Northern blot analysis has demonstrated that the NPR-C mRNA was significantly increased by PMA stimulation in HeLa cells in the presence of actinomycin D (5 μg/ml), indicating that PMA enhanced the stability of NPR-C mRNA (Fig. 1B). This result suggested that the decrease in the NPR-C mRNA level was closely associated with transcriptional suppression but not with enhanced degradation of its mRNA. However, promoter analysis using human and mouse NPR-C promoter-luciferase constructs in HeLa cells indicated that no significant decrease of transcriptional activity was observed using PMA, suggesting that the 5′-flanking regions of the human NPR-C gene (from −1332 to +13) and the mouse NPR-C gene (from −9 k to +112) were not responsible for the transcriptional regulation (data not shown).

To estimate the NPR-C protein level directly, we performed

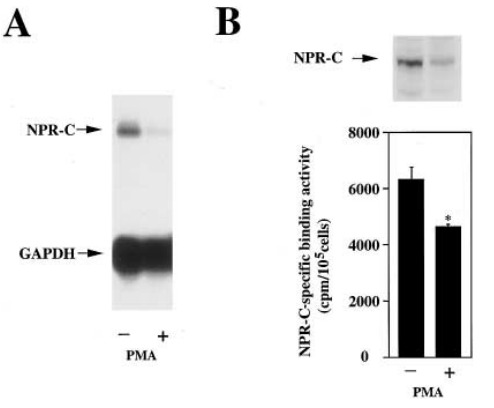

Fig. 3. Effect of a long exposure to PMA on NPR-C expression. A: HeLa cells were incubated for 18 h in the presence or absence of PMA. 5 μg of poly(A)$^+$ RNA was subjected to Northern blot analysis and hybridization was carried out with a fragment of human NPR-C cDNA as a probe. B: Western blot analysis and ANP-binding assay were carried out as described in Section 2. $^{*}P < 0.05$ compared with basal.

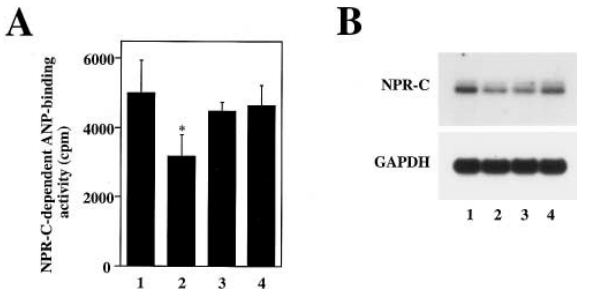

Fig. 4. Effect of protein kinase C inhibitor on NPR-C down-regulation. HeLa cells were incubated for 2 h, (lane 1) in the absence of PMA; (2) in the presence of PMA; (3) in the presence of PMA and GF109203X (0.1 μM); (4) in the presence of PMA and GF109203X (1 μM). A: HeLa cells were incubated with 0.5 nM [^{125}I]human ANP in the presence or absence of the unlabeled C-ANF(4–23) as described in Section 2. B: 5 μg of poly(A)$^+$ RNA was subjected to Northern blot analysis. *$P < 0.05$ compared with basal.

Western blot analysis using the antibody against the rat NPR-C cytoplasmic domain (Fig. 2A). At the reducing condition, the solubilized NPR-C protein was detected as a single band of approximately 63 kDa. PMA treatment for 4 h strongly reduced the level of NPR-C mRNA, and the NPR-C protein level was demonstrated to be slightly decreased by Western blot analysis (Fig. 2A). Surprisingly, the ANP-binding activity was significantly diminished by the treatment with PMA (Fig. 2B). Neither actinomycin D (5 μg/ml) nor cycloheximide (5 μg/ml) affected the reduction of ANP-binding activity following 4 h PMA treatment (Fig. 2B). These results indicate that this down-regulation of functional receptor was not dependent on reduction of its mRNA and but that post-translational mechanisms including enhanced receptor internalization are likely to be involved in the inhibitory effect of PMA treatment for 4 h on ANP-binding activity. A subsequent experiment using 18 h exposure to PMA resulted in significant decreases in mRNA and protein level (62% and 64% inhibition, respectively) in HeLa cells (Fig. 3), accompanied by a decrease in functional receptor level. These results suggest that NPR-C down-regulation after a longer exposure to PMA is attributable to transcriptional down-regulation. Furthermore, PMA-mediated transcriptional and post-translational down-regulation of NPR-C was dose-dependently blocked by the PKC-specific inhibitor GF109203X, demonstrating that PKC activation caused down-regulation of NPR-C expression through transcriptional and post-translational pathways (Fig. 4A,B). The PMA effect on ANP binding was not inhibited by the tyrosine kinase inhibitor genistein (data not shown).

4. Discussion

Here, we investigated mechanisms governing the down-regulation of NPR-C expression. In animal models as described above, serious injuries are presumed to be initiated and developed by cytokines and growth factors which are synthesized and secreted by various cell types including monocyte-macrophages, lymphocytes and vascular endothelial cells, indicating that PKC signaling was enhanced in several cells involved in pathophysiological aspects. The current study has demonstrated that PMA-activating PKC reduced the NPR-C mRNA level and NPR-C-mediated ANP-binding activity. In addition, the decrease of the NPR-C-dependent ANP-binding activity by exposure to PMA for 4 h was also produced in the presence of actinomycin D or cycloheximide, suggesting that this PMA-induced reduction was independent of de novo synthesis of new NPR-C proteins. In contrast, the inhibitory effect of PMA treatment for 18 h was paralleled by a decrease in total NPR-C protein level, suggesting that PMA-dependent reduction of functional receptor activity after a long exposure was mainly dependent on a transcriptional mechanism. The present studies demonstrated that down-regulation of NPR-C expression by PMA was mediated through two distinct pathways. Recent studies have shown that rapid down-regulation of type 1B angiotensin II receptor and basic FGF receptor was induced by PKC activation [23,24]. PMA has been demonstrated to have effects on cell function independent of the effects via PKC activation; however, PMA-induced transcriptional and post-translational down-regulation of NPR-C were effectively reversible in the presence of a specific PKC inhibitor, suggesting that PKC activation caused down-regulation of NPR-C expression through both transcriptional and post-translational pathways.

We previously cloned and characterized in detail the 5′-flanking regulatory regions of the mouse and human NPR-C genes, and AP-1 elements responsible for PMA action have been identified in the 5′-flanking regions of the mouse and human NPR-C genes [25,26]. However, transient expression analyses using NPR-C promoter-luciferase constructs in HeLa cells demonstrated no significant decrease of transcriptional activity by PMA treatment, suggesting that responsible element(s) mediating the PMA inhibitory effect were located further upstream or downstream in the NPR-C gene locus. In contrast, PMA prolonged the half-life of NPR-C mRNA, suggesting that PMA signaling blocked ribonuclease recognition. PMA has been shown to stabilize a variety of cytokine and other mRNAs, presumably through activation of PKC-mediated signal transduction cascades [27,28]. AUUUA motifs in human GM-CSF mRNA are reported to play an important role in stabilizing the mRNA by PMA treatment [29], suggesting that stabilization of the NPR-C mRNA was performed by a similar mechanism. However, full-length human NPR-C cDNA has not been isolated and, therefore, it is unclear whether the NPR-C mRNA possesses the AUUUA motif. Further experiments will be required to elucidate the PMA-dependent repression of the NPR-C gene transcription and stabilization of its mRNA.

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