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ADRENOMEDULLIN INDUCES EXPRESSION OF c-*fos* AND AP-1 ACTIVITY IN RAT VASCULAR SMOOTH MUSCLE CELLS AND CARDIOMYOCYTES

Atsuhisa Sato and Dominic J. Autelitano*

Molecular Physiology Laboratory,
Baker Medical Research Institute, P.O. Box 348 Prahran,
Melbourne, VIC, 3181, Australia

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Summary: The recently described hypotensive peptide adrenomedullin has been shown to activate various second messenger pathways in cells of the cardiovascular system though no genomic actions have yet been described. In cultured rat vascular smooth muscle cells, cardiomyocytes, and cardiac fibroblasts, adrenomedullin caused a rapid, but transient induction of c-*fos* mRNA expression in all three cell types that varied in magnitude. Adrenomedullin increased AP-1 DNA binding activities in vascular smooth muscle cells and cardiomyocytes, but not in cardiac fibroblasts. These data suggest that cardiomyocytes and vascular smooth muscle cells may be important genomic targets for this novel hormone.

AdM is a novel hypotensive peptide first isolated from human pheochromocytoma cells (1). Recent studies have shown that AdM stimulates cyclic AMP in VSMC and endothelial cells, and increases intracellular free Ca^{2+} in endothelial cells (2); though these are second messenger responses to AdM, it is uncertain whether or not AdM modulates gene transcription within cardiovascular target cells. Cyclic AMP and Ca^{2+} are well known to induce the expression of proto-oncogenes, such as c-*fos* (3). Induction of the c-*fos* gene has been associated with processes of cellular differentiation, development, and hypertrophy (3), and it has been used as a marker for the growth response in a number of systems. The product of the c-*fos* gene associates with other related proteins to form the transcription factor AP-1, which in turn mediates the transcriptional response of various so called "late-response" genes (3). Here we report that AdM induces rapid

*Corresponding author. FAX : 61-3-9521-1362.

Abbreviations: AdM, adrenomedullin; AP-1, activator protein-1; VSMC, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ANF, atrial natriuretic factor.

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and transient c-*fos* gene expression in rat VSMC, cardiomyocytes and cardiac fibroblasts, and increases AP-1 DNA binding activity in VSMC and cardiomyocytes.

Materials and Methods

Cell culture

Enriched cultures of neonatal (day 1-3) cardiomyocytes and cardiac fibroblasts were prepared from the hearts of Sprague-Dawley rats using a modified method described by Simpson et al. (4). Hearts were digested with 3 mg/ml collagenase type II (Sigma, St. Louis, MO) for 15 minutes, and incubated with 0.05% trypsin and 0.01% DNase in phosphate-buffered saline with 1 mmol/L $MgCl_2$ for 3 minutes at 37°C. Following tissue digestion, the cell suspension was incubated for 40 minutes in 6 cm tissue culture plates at 37°C, which allowed for selective attachment of nonmyocytes, most of such nonmyocyte-enriched cultures consist of fibroblasts (4). Cardiomyocyte-enriched suspensions were removed, and plated with complete medium containing 10% FCS. On the second day in culture, DNA synthesis inhibitor bromodeoxyuridine (100 µmol/L) was added. Fibroblasts were placed in DMEM containing 10% FCS. VSMC were prepared from the thoracic aortae of Sprague-Dawley rats (body weight 200 g) by an enzyme method previously described (5), and cultured in DMEM containing 10% FCS. Human AdM was purchased from Calbiochem-Novabiochem, La Jolla, CA.

Nuclease protection assays

Total RNA was isolated by acid guanidinium-phenol-chloroform method. A 219 nucleotide fragment of rat c-*fos* corresponding to nucleotides 68-287 of the published sequence (6) was subcloned into a pGEM-3Z vector for generation of cRNA probes (7). Sense RNA was synthesized in vitro from the same template and served to generate a standard curve for c-*fos* mRNA. RNA standards and samples (5 µl), 20 µl of hybridization buffer (80% formamide, 40 mmol/L PIPES pH 6.7, 0.4 mol/L NaCl, 1mmol/L EDTA) and 5 µl of antisense probe (diluted in hybridization buffer) were hybridized overnight at 45°C. Nuclease protection and RNA hybrid purification was performed as described (7). Hybrids were then analyzed on 5% non-denaturing acrylamide gels and absolute quantitation of unknown c-*fos* RNA transcripts was made by comparison with the standard curve after analysis on a Fuji BAS-1000 phosphor-imaging system.

Nuclear protein extracts

One ml ice-cold cell lysis buffer (10 mmol/L Tris,pH 8; 3 mmol/L $CaCl_2$; 2 mmol/L $MgCl_2$; 0.5 mmol/L dithiothreitol (DTT); 0.3% Triton X-100, 0.3 mol/L sucrose; 50 µg/ml leupeptin and 5 µg/ml aprotinin) was added to each well and incubated on ice for 10 minutes. Nuclei were separated by centrifugation (900 x g for 10 minutes at 4°C) as described (7), resuspended in 40 µl nuclear protein extraction buffer (20 mmol/L Tris, pH 7.5, 20% glycerol, 1.5 mmol/L $MgCl_2$, 0.3% Triton X-100, 0.5 mmol/L DTT and 0.4 mol/L KCl containing 50 µg/ml leupeptin and 5 µg/ml aprotinin) and incubated on ice for 15 minutes. The nuclear extract was then centrifuged at 15,000 X g for 10 minutes at 4°C, the supernatant removed and protein quantitated by BCA protein assay (Pierce, Rockford, Illinois, USA). Nuclear protein extracts were adjusted to 1.2 µg/µl, aliquoted and stored at -70°C.

Gel shift analysis

A canonical double stranded AP-1 oligonucleotide (5' TTC CGG CTG ACT CAT CAA GCG 3') was obtained from Promega Corp., Madison,

WI, and end labelled with T4 polynucleotide kinase to a specific activity of 2-4 X 10^8 cpm/μg. For gel shift analysis, 4 μl nuclear protein extract (4.8 μg) was combined with 4 μl 5 X shift buffer (1 X = 10 mmol/L Hepes, pH 7.5, 0.1 mmol/L EDTA, 5 mmol/L DTT, 10% glycerol and 10 μg/ml BSA), 1 μl poly [dI-dC] (1 μg) and 8 μl sterile dH_2O and pre-incubated for 5 minutes at room temperature. Following pre-incubation, 2 μl probe was added (150 pg) and incubation was continued at room temperature for a further 15 minutes. The entire reaction was then run on a 4% non-denaturing acrylamide gel with 0.5 X TBE as running buffer. DNA bound AP-1 was visualized by autoradiography and quantitated on a Fuji BAS-1000 phosphor-imaging system.

Statistical analysis

The results were expressed as mean±SEM and the statistical significance was assessed by Student's t-test. Values of $p<0.05$ were considered to be significant.

Results

Fig. 1 shows the effect of AdM on the steady state levels of c-*fos* mRNA in VSMC, cardiomyocytes, and cardiac fibroblasts. Cells were incubated in serum-free medium for 4 hours prior to treatment, and then changed to medium with or without 100 nmol/L AdM, a concentration of AdM shown to maximally stimulate cyclic AMP in VSMC and endothelial cells, and increase intracellular free Ca^{2+} in endothelial cells (2). AdM induced c-*fos* mRNA expression in all three different types of cells at 30 minutes, with the magnitude of c-*fos* induction greater in VSMC (11.8-fold; mean of three independent experiments) than those of cardiomyocytes (7.3-fold) and cardiac fibroblasts (5.5-fold).

Fig. 2 shows the time course of the effect of AdM on c-*fos* mRNA expression in VSMC, cardiomyocytes, and cardiac fibroblasts in the presence of serum. Each cell type was pre-incubated with culture medium containing freshly added 10% FCS for 1 hour, and then further treated with 100 nmol/L AdM for indicated time period. In all three cell types, addition of fresh FCS appeared to induce c-*fos* mRNA expression relative to levels in 4 hour serum starved cells (compare with Fig. 1). In VSMC, despite the elevated basal levels in the presence of FCS, c-*fos* mRNA was further induced by AdM at 30 minutes in the presence of serum, and returned to basal levels by 4 hours. A similar pattern of stimulation was observed in cardiomyocytes, although the extent of c-*fos* induction was again less than that of VSMC. In cardiac fibroblasts, c-*fos* mRNA was induced after 30 minutes incubation with 100 nmol/L AdM, and rapidly returned to basal levels at 1 hour, with the extent of c-*fos* induction both by serum or AdM minor compared with VSMC and cardiomyocytes.

To determine if the AdM-mediated induction of c-*fos* was reflected by increased AP-1 activity, we performed gel shift analysis following

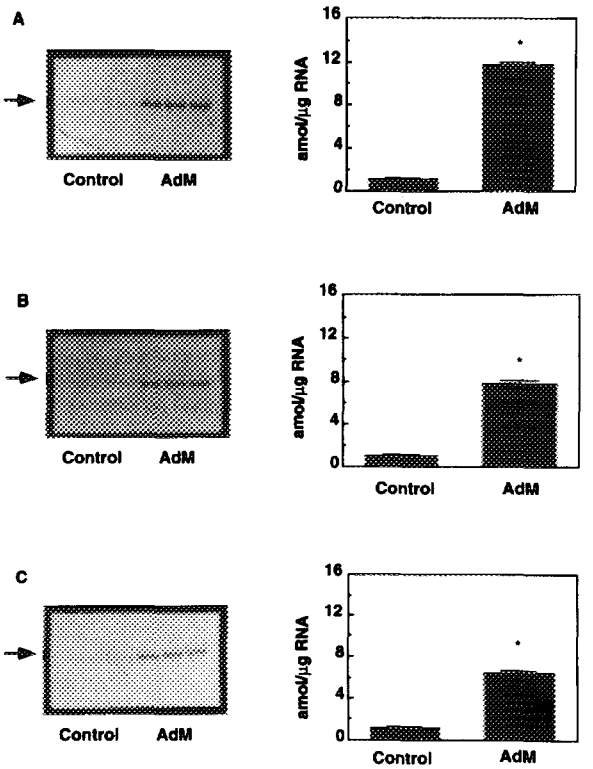
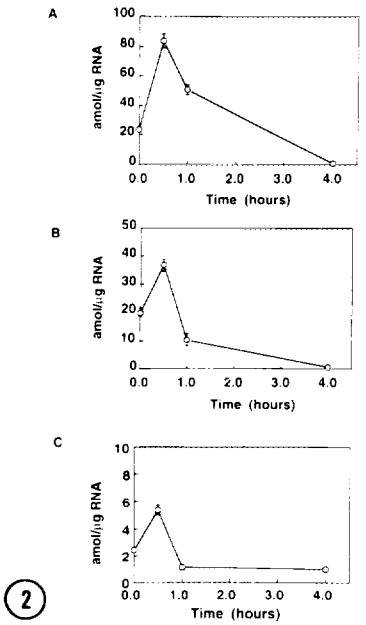
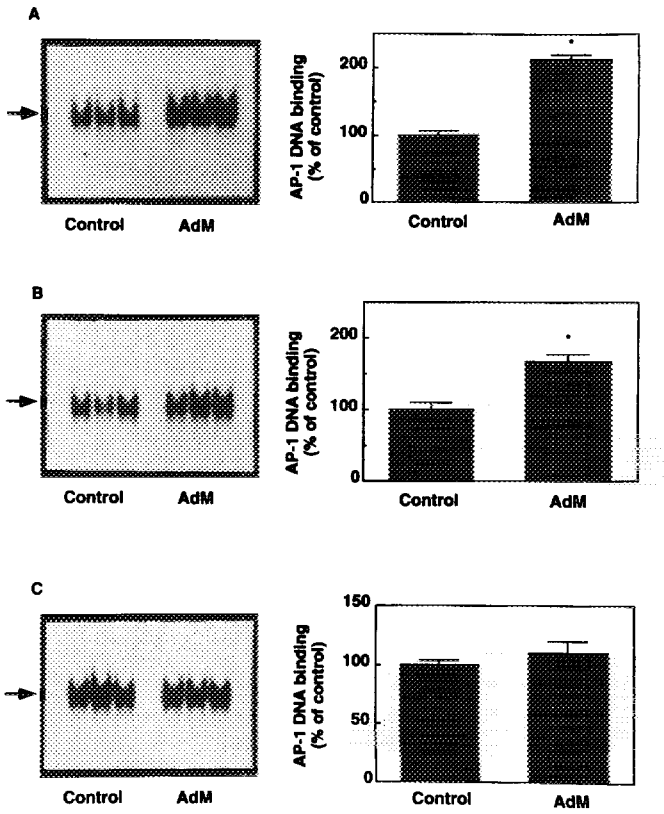


Figure 1. Effect of adrenomedullin (AdM) on c-*fos* mRNA in vascular smooth muscle cells (**A**), cardiomyocytes (**B**), and cardiac fibroblasts (**C**). Each well was incubated with serum-free medium for 4 hours before the experiment and treated with or without 100 nmol/L AdM for 30 minutes. Left panel shows autoradiogram following electrophoresis of nuclease protected c-*fos* mRNA transcripts, while the right panel shows levels of c-fos mRNA quantitated by solution hybridization and nuclease protection of 10 µg total RNA (vascular smooth muscle cells) or 15 µg total RNA (cardiomyocytes and cardiac fibroblasts). Data represent mean±SEM of three individual cultures. *$p<0.05$ vs control value.

stimulation with AdM (Fig. 3). Each well was pre-incubated with serum-free medium as described above for 2 hours, and further incubated with or without 100 nmol/L AdM for 2 hours. AP-1 DNA binding activity was detected in basal extracts of all three cell types. AdM significantly increased AP-1 DNA binding activity in VSMC (212 ± 9% of control cells) and cardiomyocytes (167 ± 9% of control cells) at the time point measured. No increase in AP-1 DNA binding activity was observed in cardiac fibroblasts after 2 hour incubation with 100 nmol/L AdM. AP-1 DNA binding activities both in control and AdM-treated cells could be inhibited by excess of cold AP-1 DNA, but not by an unrelated DNA element (SP-1), confirming the specificity of the protein-DNA interaction (data not shown).



(2)



(3)

Figure 2. Time course for the effect of AdM on c-*fos* mRNA in vascular smooth muscle cells (**A**), cardiomyocytes (**B**), and cardiac fibroblasts (**C**). Each well was preincubated with culture medium containing 10% FCS for 1 hour and further treated with 100 nmol/L AdM for indicated time period. Levels of c-*fos* mRNA were quantitated by solution hybridization and nuclease protection of 10 μg total RNA. Data represent mean±SEM of three individual cultures at each time point.

Figure 3. Gel shift analysis of AP-1 DNA binding activity in nuclear protein extracts from vascular smooth muscle cells (**A**), cardiomyocytes (**B**), and cardiac fibroblasts (**C**). Each well was preincubated with serum-free medium for 2 hours and further incubated with or without 100 nmol/L AdM for 2 hours. Nuclear protein extracts (4.8 μg/reaction) were used in gel shift analysis using a consensus AP-1 DNA probe (TGACTCA). Left hand panel shows AP-1 DNA binding activity after electrophoresis and autoradiography, while right hand panels represent quantitation of AP-1 binding. Data represent mean±SEM of three individual cultures. *$p<0.05$ vs the value in the control experiment.

Discussion

The present study has shown that AdM induces rapid and transient c-*fos* gene expression in VSMC, cardiomyocytes and cardiac fibroblasts, and increases AP-1 DNA binding activity in VSMC and cardiomyocytes. These findings strongly suggest that AdM may directly regulate cell differentiation or proliferation in these cells through c-*fos* induction, and

our study also suggests that within the heart cardiomyocytes can be considered to be an important target of AdM. The clinical implications of proto-oncogenes expression in the cardiovascular system have mainly focused on the relationship with abnormal VSMC and/or cardiomyocyte growth. In both cell types, several peptides and growth factors have been shown to induce c-*fos* gene expression (8), and are considered to be involved in the process of abnormal VSMC growth or cardiac hypertrophy. Moreover, in heart, c-*fos* induction and subsequent increases in AP-1 DNA binding activity also could mediate the increase of ANF secretion and gene expression from myocytes (9). Given the potent hypotensive effect of AdM, the potential relationship with ANF seems to be of particular importance. In conclusion, AdM induces rapid and transient c-*fos* gene expression in VSMC, cardiomyocytes and cardiac fibroblasts, and increases AP-1 DNA binding activity in VSMC and cardiomyocytes. These results suggest the possibility of a novel mechanism of AdM action on cells of the cardiovascular system, and suggest that the AdM-mediated induction of c-*fos* and AP-1 may be involved in the transcriptional regulation of various target genes in these cells, that may in turn lead to longer-term changes in cellular phenotype.

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