Arginine vasopressin stimulates proliferation of adult rat cardiac fibroblasts via protein kinase C-extracellular signal-regulated kinase 1/2 pathway

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Abstract: Arginine vasopressin (AVP), a neurohormone and hemodynamic factor implicated in the pathophysiology of hypertension and congestive heart failure, can also act as a growth-stimulating factor. Our previous work demonstrated that AVP is a mitogen for neonatal rat cardiac fibroblasts (CFs). In the present study, we extended our investigations to adult rat CFs to explore whether AVP could induce adult rat CF proliferation and, if so, to identify the mechanism involved. Adult rat CFs were isolated, cultured and subjected to AVP treatment. DNA synthesis and cell cycle distribution were analyzed by [3H]-thymidine incorporation and flow cytometry. Cellular extracellular signal-regulated kinase 1/2 (ERK1/2) activity was measured by in vitro kinase assay using myelin basic protein (MBP) as a substrate. Protein expressions of total- and phospho-ERK1/2, p27 Kip1, cyclins D1, A, E were assessed by Western blot. The results showed that AVP stimulated DNA synthesis in adult rat CFs, and the effect was abolished by a V1 receptor antagonist, d(CH2)5[Tyr2(Me), Arg8]-vasopressin (0.1 µmol/L), but not by a V2 receptor antagonist, desglycinamide-[d(CH2)5, D-Ile2, Ile4, Arg8]-vasopressin (0.1 µmol/L). AVP induced an activation of ERK1/2, which could be mimicked by the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA, 30 nmol/L, 5 min), but abolished by depletion of PKC via chronic PMA incubation (2.5 µmol/L, 24 h). In addition, AVP down-regulated protein expression of p27Kip1, increased protein expressions of cyclins D1, A and E, and induced cell cycle progression from G0/G1 into S stage. Inhibition of ERK1/2 activation by PD98059 (30 µmol/L) abolished the effect of AVP on DNA synthesis, protein expressions of p27Kip1, cyclins D1, A and E as well as cell cycle progression. These results suggest that AVP is also a growth factor for adult rat CFs. The mitogenic effect of AVP is mediated via V1 receptors and PKC-ERK1/2 pathway. Moreover, AVP modulates the expressions of cell cycle regulatory proteins p27Kip1 and cyclins D1, A and E, which lie downstream of ERK1/2 activation, and induces cell cycle progression in adult rat CFs.

Key words: arginine vasopressin; V1 receptor; extracellular signal-regulated kinase 1/2; protein kinase C; p27Kip1; cyclin; cardiac fibroblast

蛋白激酶 C- 细胞外信号调节激酶 1/2 通路介导精氨酸升压素对成年大鼠心肌成纤维细胞的促增殖作用

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摘要: 精氨酸升压素arginine vasopressin, AVP)是高血压和心力衰竭时激活的神经体液和血流动力学因子，同时，它还具有直接的生长刺激作用。我们以往的研究显示 AVP 可诱导新生大鼠心肌成纤维细胞(cardiac fibroblasts, CFs)增殖。本研究旨在进一步观察 AVP 是否对成年大鼠 CFs 具有促增殖作用，并探讨其机制。采用组织块法培养成年大鼠 CFs，用[3H]-TdR 播入法和流式细胞仪方法观察 AVP 作用下 CFs 的 DNA 合成和细胞周期分布。根据特异性底物髓磷脂基质蛋白(myelin basic protein, MBP)的磷酸化水平测定细胞外信号调节激酶 1/2 (extracellular signal-regulated kinase 1/2, ERK1/2) 的活性。用 Western blot 检测 ERK1/2 的磷酸化和 p27Kip1, 细胞周期蛋白 D1, A, E 的表达。结果发现，AVP (0.1 µmol/L) 可促进成年大鼠 CFs 的 DNA

Received 2008-01-15 Accepted 2008-02-25
This work was supported by the Natural Science Foundation of Shaanxi Province, China (No. 2004C2-21).
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Adverse cardiac remodeling is characterized by interstitial fibrosis, cardiac myocyte death or hypertrophy and proliferation of cardiac fibroblasts (CFs) [1-3]. Despite its adaptive nature, cardiac hypertrophy is independently associated with increased risks in cardiovascular morbidity and mortality. It has been critically implicated in the pathophysiology of chronic congestive heart failure, which features a worsening neurohormonal profile. As a neurohormone and hemodynamic factor, elevated plasma arginine vasopressin (AVP) has been documented in both hypertension and congestive heart failure [4,5]. Sustained and chronic activation of AVP system, as triggered by congestive heart failure, leads to progressive cardiac remodeling [6]. AVP can also act as a potent growth-stimulating factor for a variety of cell types, including mesangial cells [7], hepatocytes [8], osteoblast-like cells [9], and intestinal epithelial cells [10]. CFs account for up to two-thirds of the total cells in the normal heart and are responsible for maintaining its structural integrity through controlled proliferation and extracellular matrix production. Our previous work showed that AVP induces pro-fibrotic response in neonatal rat CFs [11]. Importantly, AVP also induces neonatal rat CF proliferation via the V1 receptor [12]. However, the mechanism for this mitogenic effect is less well-defined. In the present study, we extended our investigations to adult rat CFs to explore whether AVP could induce adult rat CF proliferation and, if so, to provide further insight into the underlying mechanism.

1 MATERIALS AND METHODS

1.1 Culture and characterization of adult rat CFs

All the experiments were carried out under the regulations of the National Institutes of Health Guidelines on the Use of Laboratory Animals. CFs from adult Sprague-Dawley (SD) rats (200-250 g, male, from the Experimental Animal Center of the Fourth Military Medical University) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated in a humidified atmosphere of 5% CO2 at 37 °C. Immunocytochemical examinations showed that all cultured cells exhibited positive staining for vimentin (indicating origin of mesenchyma), but negative for von Willebrand’s factor (indicating little contamination of vascular endothelial cells) and α-smooth muscle actin (indicating little contamination of smooth muscle cells). Subconfluent cells were made quiescent in serum-free DMEM for 24 h. The first passage cells were used in the present study.

1.2 [3H]-thymidine incorporation

Adult rat CFs were seeded on 96-well plates at a density of 5×10³ cells/well for 24 h in 10% FBS/DMEM, then starved and exposed to various concentrations of AVP or/and an inhibitor of the upstream kinase of extracellular signal-regulated kinase 1/2 (ERK1/2), PD98059, for 24 h. DNA synthesis was assessed by the addition of 1 μCi/mL of [3H]-thymidine (Atomic Energy Institute, Chinese Academy of Science) 6 h prior to the end of the treatment period. Cells were then washed twice with cold PBS (4 °C), and incubated with 4 °C 10% trichloroacetic acid (TCA) for 30 min. The precipitates were rinsed twice with 95% ethanol and solubilized in 0.5 mol/L NaOH. The radioactivity in aliquots was measured in a liquid scintillation spectrometer (Beckman DU640, USA).

1.3 Cell cycle analysis

2×10⁴ CFs were plated in 100 mL cell culture flasks and incubated in DMEM containing 10% FBS for 18 h. After starvation, cells were treated with DMEM containing 2.5% FBS in the presence or absence of AVP or/and PD98059 for 24 h. Cells were detached with 0.25% trypsin and fixed with 70% ethanol. Cell cycles were analyzed with flow cytometry after rinsing with PBS.

1.4 ERK1/2 activity assay

ERK1/2 activity was measured by using myelin basic
protein (MBP) as a substrate, as described previously\(^{1(3)}\) with modification. After AVP treatment for intervals of 2, 5, 10, 30, 60 and 120 min, CFs were washed twice with ice-cold PBS and lysed with 0.5 mL lysis buffer, which contained 2 mmol/L EDTA, 20 mmol/L HEPES (pH 7.2), 5 mmol/L EGTA, 20 mmol/L β-glycerophosphate, 20 mmol/L NaF, 50 mmol/L NaCl, 0.2 mmol/L Na2VO4, 0.01% Triton X-100, and 0.5 mmol/L fresh phenylmethylsulfonyl fluoride. Cell lysates were prepared by freezing in liquid nitrogen, thawing on ice, scraping, and sonicating (5 s). After centrifugation for 10 min (10 000 × g, 4 ºC), 5 μL supernatant of the extract was added to 30 μL of reaction buffer containing 20 mmol/L HEPES (pH 7.2), 10 mmol/L MgCl2, 2 mmol/L MnCl2, 0.5 mmol/L Na2VO4, 2 mmol/L dithiothreitol, 2 mmol/L TTTAAPIASGATGAAAIAH (cAMP-dependent protein kinase inhibitor peptide, Sigma, USA), 7.4×104 Bq [32P] ATP and 25 μg MBP. After incubation at 30 ºC for 15 min, the reaction was stopped by adding 10 μL of stopping solution containing 0.6% HCl, 1 mmol ATP, and 1% bovine serum albumin. 20 μL of the reaction mixture was spotted on 1.5 cm × 1.5 cm squares of P81 phosphocellulose filter paper, washed three times with deionized water containing 1% orthophosphoric acid, and dried. Radioactivity was then measured by scintillation counting. ERK1/2 activity was expressed as fold increase compared to the control.

1.5 Western blot analysis

CFs were treated with AVP or/and PD98059 for 5 min (for total- and phospho-ERK1/2) or 24 h (for p27\(^{38w}\), cyclins D1, A and E) and lysed with the same lysis buffer as mentioned above. Protein content was measured by Bradford assay with Bio-Photometer (BioPhotometer 6131 GB/HK, Eppendorf) at 595 nm. Equal amount of proteins in each sample were separated by SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane, and incubated in 5% defatted milk dissolved in TBS (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20). Primary antibodies against p27\(^{38w}\), cyclins D1, A, E, total- and phospho-ERK1/2 (Sigma, USA) were diluted in TBS using the appropriate dilutions. The membranes were probed with the indicated primary antibodies and followed by the proper secondary horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz; diluted from 1:3 000 to 1:10 000 in TBS). The membranes were then washed 4 times with TBS at room temperature. To re-probe another primary antibody, membranes were incubated in stripping buffer (62.5 mmol/L Tris, pH 6.7, 100 mmol/L of 2-mercaptoethanol, and 2% SDS) for 30 min, washed, and then used for further study. The reactive proteins were visualized using enhanced chemiluminescence (ECL).

1.6 Statistical analysis

Data were presented as mean±SEM. Comparisons were conducted using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. P<0.05 was considered statistically significant.

2 RESULTS

2.1 AVP stimulates DNA synthesis in adult rat CFs via V1 receptor

Consistent with previous work in our laboratory in neonatal rat CFs\(^{1(2)}\), AVP (0.001-1 μmol/L) increased DNA synthesis as measured by [3H]-thymidine incorporation in adult rat CFs in a dose-dependent manner (Fig.1A). And 0.1 μmol/L was chosen as AVP working concentration for subsequent analysis. Further investigation revealed that the effect of AVP on DNA synthesis was abolished by incubating adult rat CFs with a V1 receptor antagonist, d(CH\(_2\)_3[Tyr\(^{2}(Me)\), Arg\(^{8}\)]vasopressin (0.1 μmol/L), but not by a V2 receptor antagonist, desglycaminamide-[d(CH\(_2\)_3), D-Ile\(^{6}\), Ile\(^{7}\), Arg\(^{8}\)]vasopressin (0.1 μmol/L) (Fig.1B), indicating that the growth-stimulating effect of AVP on adult rat CFs is mediated via the V1 receptor.

2.2 PKC-ERK1/2 pathway is involved in AVP-induced DNA synthesis in adult rat CFs

To explore the potential involvement of ERK1/2 activation, which is known to mediate the mitogenic response induced by growth-stimulating factors in many cell types, we first measured ERK1/2 activity in adult rat CFs exposed to AVP. ERK1/2 activity assay showed that cellular ERK1/2 activation could readily be induced by AVP (0.1 μmol/L). The activation was detectable at 2 min (1.5-fold induction), peaked at 5 min (2.7-fold induction) and sustained through 1 h, with subsequent decline nearly to baseline level at 2 h after the initiation of stimulation (Fig.2A).

Since the PKC-ERK1/2 pathway has been implicated in the mitogenic effect of AVP on rat intestinal epithelial cells\(^{1(0)}\), we further tested the possible involvement of PKC-ERK1/2 pathway in AVP-induced adult rat CF proliferation. Incubating CFs with an inhibitor of the upstream kinase of ERK1/2, PD98059 (30 μmol/L), abolished the mitogenic effect of AVP, while the basal cell DNA uptake was not significantly altered (Fig.2B). Exposing cells to the pharmacological PKC activator, phorbol 12-myristate 13-acetate (PMA, 30 nmol/L), as well as AVP for 5 min activated ERK1/2 phosphorylation as measured by Western blot.
Fig. 2. PKC-ERK1/2 pathway is involved in AVP-induced DNA synthesis in adult rat CFs.

**A**: CFs were incubated with AVP (0.1 μmol/L) for the indicated time. The activity of ERK1/2 was measured by myelin basic protein (MBP) phosphotransferase activity.

- *P* < 0.05 vs the baseline level.

**B**: [3H]-thymidine incorporation into cells treated with AVP (0.1 μmol/L) alone for 24 h or in the presence of PD98059 (PD, 30 μmol/L) for 24 h before and during AVP stimulation. #P < 0.05 vs AVP.

**C**: Western blot analysis of phospho- and total ERK1/2 protein expressions in cells exposed to PMA (2.5 μmol/L) for 24 h before and during AVP stimulation. #P < 0.05 vs AVP.

Depleting PKC by chronic PMA incubation (2.5 μmol/L, 24 h) abolishes the stimulating effect of AVP on ERK1/2 phosphorylation (Fig. 2C), suggesting that the observed ERK1/2 activation induced by AVP in adult rat CFs is PKC-dependent.

**2.3 p27Kip1 and cyclins D1, A and E are the downstream targets of ERK1/2 in mediating AVP-induced DNA synthesis in adult rat CFs**

Next, we explored the downstream effectors of ERK1/2 in mediating AVP-induced DNA synthesis in adult rat CFs. Western blot analysis revealed that the protein expression of p27Kip1, which negatively regulates mammalian cell cycle progression, was markedly attenuated upon AVP treatment, accompanied by increased expressions of cyclins D1, A and E. Consistent with the changes observed in DNA synthesis, inhibiting ERK1/2 activation by PD98059 (30...
μmol/L) abolished the effect of AVP on protein expressions of p27<sup>Kip1</sup> as well as cyclins D1, E and A (Fig.3).

### 2.4 AVP induces cell cycle progression in adult rat CFs and ERK1/2 is involved

To gain a more direct insight into whether AVP induces cell cycle progression in adult rat CFs, we employed flow cytometry to analyze cell cycle distribution. Cell growth was synchronized by serum starvation. As shown in Table 1, AVP induced cell cycle progression from G<sub>0</sub>/G<sub>1</sub> into S phase, accompanied by increased proliferation index (PI). The effects of AVP on G<sub>0</sub>/G<sub>1</sub>-S phase transition and PI were inhibited by PD98059 (30μmol/L), which alone was insufficient to induce any detectable change in cell cycle distribution and PI value. These results indicate that ERK1/2 is

![Fig. 3](image)

**Table 1.** Cell cycle distribution in adult rat CFs incubated with AVP or/and PD98059

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell cycle distribution (%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G&lt;sub&gt;0&lt;/sub&gt;/G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>86.75±1.27</td>
<td>8.72±1.07</td>
</tr>
<tr>
<td>0.1 μmol/L AVP</td>
<td>70.50±1.35</td>
<td>18.53±1.08*</td>
</tr>
<tr>
<td>0.1 μmol/L AVP + 30 μmol/L PD98059</td>
<td>84.07±1.37</td>
<td>10.40±1.20*</td>
</tr>
<tr>
<td>30 μmol/L PD98059</td>
<td>87.05±1.25</td>
<td>8.57±0.82</td>
</tr>
</tbody>
</table>

Mean±SEM, n=6. Proliferation index (PI) = S+G<sub>2</sub>/M. *P<0.05 vs control, †P<0.05 vs AVP.
indispensable for cell cycle progression through G1 phase and the subsequent cellular proliferation.

3 DISCUSSION

The chronic elevation of AVP levels in patients with hypertension or heart failure ultimately leads to detrimental myocardial effects. For example, the survival and ventricular enlargement (SAVE) trial has demonstrated that high AVP levels are associated with poor long-term prognosis[15], which might indicate its role not only in the pathophysiology of the heart failure but also in disease progression. Thus AVP has emerged as an important candidate for therapeutic intervention in the treatment of cardiac hypertrophy.

A large pool of studies have suggested that angiotensin II and endothelin 1, two of the neurohormoral factors increased in patients with hypertension or congestive heart failure apart from AVP, directly induce cardiac hypertrophy independent of hemodynamic mechanisms. Although AVP plays an important role in the development of cardiac hypertrophy via increased peripheral vascular resistance and increased cardiac afterload, the direct role of AVP in cardiac hypertrophy is not firmly established. In rat models, AVP has been shown to act as a growth factor and induce cardiomyocyte hypertrophy[16-18]. In these studies, AVP was found to have a direct protein synthetic effect on cultured cardiomyocytes with motivation of calcium and activation of mitogen-activated protein kinase (MAPK). However, the signaling events and cellular effects of AVP with respect to CFs are not well defined. CF is the major cell type that controls cardiac remodeling under physiological and pathologic conditions[9]. Unlike myocytes, CFs are highly proliferative and sensitive to numerous hormonal factors. Combining the present work with our previous observation[21], we demonstrated that AVP is a potent mitogen for both neonatal and adult rat CFs. The mitogenic effect on adult rat CFs was mediated via V1 receptors and PKC-ERK1/2 pathway. Further examination revealed that ERK1/2 activation decreased protein expression of cell cycle inhibitor p27Kip1 and increased expressions of cyclins D1, E and A, thus identified the cell cycle regulatory proteins linking the PKC-ERK1/2 pathway activation and the proliferation of adult rat CFs.

The AVP receptors can be categorized into V1 and V2 receptors. The binding of AVP to the V1 receptor is known to stimulate the degradation of inositol phospholipids, to produce the intracellular second messengers diacylglycerol, which activates PKC, and phosphatidylinositol 4,5-bisphosphate, which mobilizes Ca2+. The downstream messengers play pivotal roles in a variety of cell type-specific responses. Although AVP has been shown to activate ERK1/2 in rat cardiomyocytes[20,21], the effect of AVP on ERK1/2 activation in rat CFs has not been reported. In the present study, we demonstrated that AVP activates ERK1/2 in adult rat CFs and this stimulation is mediated through the V1 receptor and PKC. Interestingly, however, AVP-induced cardiomyocyte growth can also be mediated via the PKC-ERK1/2 pathway[21], which, in line with our present work, might indicate the significance of this pathway in regulating the growth of cardiac cells.

Mitogenic signaling through ERK1/2 induces cell cycle entry in many cells, including fibroblasts[22] and smooth muscle cells[23]. ERK, a subfamily of MAPK, is a critical regulator of cell cycle progression into S phase. Cyclin D is a rate-limiting, step-controlling initiator of DNA replication whose transcription initiation, assembly, and nuclear transport is mitogen-dependent. In the present study, we demonstrated that ERK1/2 activation plays a pivotal role in the induction of cyclin D1 expression upon AVP treatment. This is in agreement with previous observations showing that the extent of cyclin D1 accumulation is correlated with the mitogenic potential of growth factors and their ability to induce ERK1/2 activation[21].

The protein p27Kip1 is a cyclin-dependent kinase inhibitor that regulates cell cycle progression. It has been established that p27Kip1 is an essential component of the pathway that connects mitogenic signals to the cell cycle at the restriction point in fibroblast cell cycle progression[20]. Several studies have indicated that ERK1/2 activation contributes to the down-regulation of p27Kip1[26-28]. However, most of these observations have been focused on cancer cells. By using flow cytometry to analyze cell cycle distribution, it was shown that AVP induced the G1/S phase transition in adult rat CFs, which is also a highly proliferative cell type. We also confirmed a role of endogenous ERK1/2 activation in G1 phase progression via the inhibition of p27Kip1 expression. Moreover, the expression of cyclin E, which is involved in late G1 phase progression, and cyclin A, which is required for the onset of DNA replication during S phase[20], was markedly induced by AVP stimulation via the mediation of ERK1/2.

Taken together, our findings demonstrated that AVP acts as a potent mitogen for adult rat CFs and elucidated the functional role of PKC-ERK1/2 pathway in mediating this mitogenic effect. We also identified the essential targets of ERK1/2 and characterized their cell cycle-related effects. An understanding of the effects of AVP on CF proliferation and the underlying mechanism may provide useful insight.
into the study and treatment of adverse cardiac remodeling, which is deeply implicated in the pathophysiology of hypertension and congestive heart failure.

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