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Vasopressin up-regulates the expression of growth-related immediate-early genes via two distinct EGF receptor transactivation pathways

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Abstract

Activation of V_{1a} receptor triggers the expression of growth-related immediate-early genes (IEGs), including c-Fos and Egr-1. Here we found that pre-treatment of rat vascular smooth muscle A-10 cell line with the EGF receptor inhibitor AG1478 or the over-expression of an EGFR dominant negative mutant (HEBCD533) blocked the vasopressin-induced expression of IEGs, suggesting that activation of these early genes mediated by V_{1a} receptor is via transactivation of the EGF receptor. Importantly, the inhibition of the metalloproteinases, which catalyzed the shedding of the EGF receptor agonist HB-EGF, selectively blocked the vasopressin-induced expression c-Fos. On the other hand, the inhibition of c-Src selectively blocked the vasopressin-induced expression of Egr-1. Interestingly, in contrast to the expression of c-Fos, the expression of Egr-1 was mediated via the Ras/MEK/MAPK-dependent signalling pathway. Vasopressin-triggered expression of both genes required the release of intracellular calcium, activation of PKC and β -arrestin 2. These findings demonstrated that vasopressin up-regulated the expression of c-Fos and Egr-1 via transactivation of two distinct EGF receptor-dependent signalling pathways.

Keywords

Vasopressin; PKC; mitogen-activated kinases; immediate early genes; EGFR transactivation; metalloproteinases

1. Introduction

Arginine vasopressin (AVP) plays a central role in the mechanisms regulating blood pressure by stimulating the contraction of vascular smooth muscle cells and water reabsorption in the kidney [1–7]. Importantly, AVP acts as a growth factor inducing hypertrophy and cell growth in a variety of cell types [8–13]. AVP-stimulated cellular responses are mediated by three AVP receptors subtypes (V₁, V₂ and V₃), which belong to the superfamily of G-protein-coupled

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receptors (GPCRs). Like many GPCRs, the V₁ receptor transactivates the EGF receptor (EGFR) to induce the expression of immediate early genes leading to the cell cycle progression and growth [14–19]. GPCRs transactivate EGFR via several mechanisms [20,21]. One mechanism involves the activation of a membrane-bound metalloproteinase that catalyzes the extracellular shedding of HB-EGF, which then activates the EGFR [22–25]. A second mechanism involves the activation of c-Src, which leads to the phosphorylation and activation of EGFR [26–28]. Additionally, tyrosine kinase receptors can use GPCR-mediated signalling pathways to stimulate downstream effectors, such as ERK1/2 [29]. This mechanism of cross-talk between tyrosine kinase receptors and the GPCRs has been designated as integrative signalling [29,30]. Since the growth of the smooth muscle cell is important for the arterial wall stiffness and for the onset of hypertension, we investigated the mechanisms of the AVP triggered-expression of two growth-related genes c-Fos and the early growth response gene 1 (Egr-1) in A-10 cells. This cell line is derived from rat smooth muscle cells, which endogenously express both V₁ and EGF receptors. In this work we showed that AVP-induced up-regulation of c-Fos and Egr-1 is mediated by the stimulation of two distinct EGFR transactivation mechanisms.

2. Material and methods

2.1 Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, glutamine, and fungizone were from Invitrogen. Phorbol, 12-myristate, 13-acetate, GF109203X, PD98059, Y27632, PP1 and AG 1478 were from Calbiochem. GM6001 was from Chemicon. MMP Inhibitor III was from Merck. Ultraspec was from Biotecx. Pertussis toxin was from Biomol International. Antibody against phospho-retinoblastoma protein was from Cell Signaling Technology, anti-Egr-1 and anti c-Fos were from Santa Cruz Biotechnology. The V₁ antagonist d(CH₂)₅[Tyr²(Me)Tyr⁹(NH₂)]AVP was kindly provided by Prof. M. Manning (Medical College of Ohio, Toledo, USA). The siRNA for β -arrestin 2 was purchased from Invitrogen.

2.2. Expression vectors

Plasmids encoding wild type c-Src and c-SrcK295R/Y527F were a generous gift from Dr. Joan S. Brugge (Harvard Medical School, USA). The plasmid encoding L61S186Ras was generously provided by Dr. Kun-Liang Guan (University of Michigan Medical School, USA). The EGFR dominant negative mutant HERCD533 was generously provided by Dr. S Meloche (University of Montreal, Quebec, Canada). The plasmid encoding the C-terminus of β -adrenergic receptor kinase (CT- β GRK2) was a generous gift from Dr. Juan Olate (Universidad de Concepción, Chile). The plasmid encoding the S1 catalytic subunit of Pertussis toxin was kindly provided by Dr. Halvard Bonig (University of Washington, USA).

2.3. Cell culture and transfections

The smooth muscle cell line A-10 (ATCC CRL 1476) was cultured to subconfluency on 35 mm dishes in DMEM containing 10% FBS. Serum starved cells were treated with vasopressin in the absence and presence of inhibitors. The reaction was stopped by addition of 100 μ l of ice-cold RIPA buffer (150 mM NaCl, Tris/ HCl pH 8.0, 1% deoxycholic acid, 1% Nonidet P40, 0.1% SDS, 4 mM EDTA, 1 mM Na₃VO₄, 250 μ g/ml p-nitrophenyl phosphate, 1 mM phenylmethane-sulphonyl fluoride, 1 μ g/ml each of leupeptin, pepstatin A and aprotinin). Cells were lysed and proteins were precipitated by addition of trichloroacetic acid and resuspended in electrophoresis sample buffer containing 1 mM Na₃VO₄. In some experiments, cells were incubated with the V₁ antagonist d(CH₂)₅[Tyr²(Me)Tyr⁹(NH₂)]AVP, GF109203X or with PD98059 or with AG 1478 or with MMP or GM6001 inhibitors prior the stimulation with AVP. Transient transfections were carried out using FuGENE 6 Transfection Reagent (Roche

Diagnostics). The siRNA for β arrestin 2 was transfected using Block-iT Transfection Kit (Invitrogen).

2.4 Western blotting

Cell extracts were fractionated using SDS-PAGE, and the proteins were electrotransferred onto nitrocellulose filters using 0.05% SDS in the transfer buffer (20 mM Tris-glycine pH 8.3 and 20% methanol). Blots were incubated with anti c-Fos or anti Egr-1 or anti phospho-retinoblastoma protein antibodies at a dilution of 1:1,000. The blots were then incubated with peroxidase-labeled secondary antibody at a dilution of 1:50,000 followed by chemiluminescence.

2.5. Reverse Transcription and Polymerase Chain Reaction

Total RNA from A-10 cells was prepared using Ultraspec (Biotecx). cDNAs were synthesized using oligo dt primers and SuperScript II (Gibco BRL). PCR were carried out using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal non-competitive standard to normalize the expression of the gene to be studied. The pair of primers for the c-Fos were the sense 5'GCCAACTTTATCCCCACG3' and the anti-sense 5'TAGAAGGAACCAGACAGGTCC3' which should generate a fragment of 725 bp, for the Egr-1 were the sense 5'-CTTCAGTCGTAGTGACCACCT-3' and the anti-sense 5'-ATGTCTGAAAGACCAGTTGAG-3' which should generate a fragment of 441 bp, and for the GAPDH were the sense 5'TCCCTCAAGATTGTCAGCAA-3 and the anti-sense 5'-AGATCCACAACGGATACATT-3" which should generate a fragment of 309 bp. The ratio of the co-amplification of products was estimated at the exponential phase of the PCR. The PCR reaction mixture contained 50 pMol of each primer for gene to be studied and for GAPDH, 1 mM deoxynucleotides 1x Taq polymerase reaction buffer, 1.5 mM MgCl₂ and 2.5 U Taq polymerase. PCR products were fractionated by electrophoresis on 1.6 % agarose gel, stained with ethidium bromide and the bands visualized with a UV transilluminator.

3. Results

3.1. The V_{1a} Receptor Mediated the Expression of IEGs

Because AVP triggers cell proliferation in several cell types, we initially tested the AVP-induced expression of genes regulating cell growth in the aorta smooth muscle derived A-10 cell line. Western blot analysis showed that AVP up-regulated the expression of genes regulating cell growth, including cyclin D1 and the immediate early genes c-Fos and Egr-1 (Fig. 1). In addition, we showed the AVP-dependant phosphorylation of the retinoblastoma protein (Rb), which is a essential in the cell cycle progression. RT-PCR assays demonstrated that the AVP-dependant expression of c-Fos was transient, reaching a maximum level at 30 min; whereas the expression of Egr-1 was sustained for up to 3h (Fig. 2A). Interestingly, EGF-triggered expression of both c-Fos and Egr-1 was fast and transient (Fig. 2B). To identify the AVP receptor subtype responsible for the up-regulation of these early immediate genes we employed AVP receptor-specific antagonists. We found that the V_{1a} antagonist d(CH₂)₅[Tyr²(Me)Tyr⁹(NH₂)]AVP blocked the AVP-induced up-regulation of both IEGs (Fig. 3), suggesting that their expression is mediated via the V_{1a} receptor subtype. The V₂ specific agonist, desmopressin, did not affect the expression of IEGs in A-10 cells (Data not shown).

3.2. AVP-induced up-regulation of IEGs required activation of PKC, intracellular calcium and β -arrestin 2

A-10 cells depleted of PKC by the long-treatment with phorbol esters failed to elicit AVP-dependant expression of both c-Fos and Erg-1 (Fig. 4A). Similarly, the PKC inhibitor Gö6983 blocked the AVP-induced up-regulation of both genes (Fig. 4B). In contrast, the PKC inhibitor

GF109203X (GFX) was a weak inhibitor of the AVP-dependent up regulation of these genes (Data not shown). Further, cells treated with the intracellular calcium chelator BAPTA failed to respond to AVP-induced up-regulation of IEGs (Fig. 4C), suggesting that the release of intracellular calcium is required for the AVP effect.

Since the activated and phosphorylated V_1 receptor binds β -arrestin 2, desensitizing the responses mediated by the receptor and stimulating non-G protein signalling pathways [31, 32], we examined the role of β -arrestin 2 in the AVP-induced expression of IEG. We showed that A-10 cells depleted of β -arrestin 2 by specific siRNA failed to elicit AVP-induced up-regulation of both IEGs (Fig. 4D). This finding indicates that β -arrestin 2 is required in the AVP-induced up-regulation of IEG.

3.3. AVP-induced up-regulation of c-Fos and Egr-1 via two distinct EGFR transactivation pathways

We employed the EGFR kinase inhibitor AG1478 and cells over-expressing the EGFR dominant negative mutant HERCD533 to determine whether AVP-stimulated the expression of c-Fos and Egr-1 involves activation of the EGF receptor. Fig 5A and 5B showed that AG1478 or the over-expression of HERCD533 blocked the AVP-induced up-regulation c-Fos and Egr-1. These results indicate that AVP-induced expression of both genes required activation of the EGF receptor in A-10 cells.

To determine whether the mechanism of EGFR transactivation mediated by the V_{1a} receptor is via the shedding of the EGF receptor agonist HB-EGF, we employed two different metalloproteinase inhibitors (MMP-II and GM6001) of the A Disintegrin And Metalloproteinase (ADAM), which cleaves the pro-HB-EGF to release HB-EGF [33]. We showed that MMP-II (Fig. 6A) and GM6001 (Fig. 6B) blocked selectively the AVP-induced up-regulation of c-Fos. These inhibitors did not affect the expression of Egr-1. Similarly, we found that the Rho kinase (ROCK) inhibitor Y27632 abrogated the AVP-mediated up-regulation of c-Fos without affecting the expression of Egr 1 (Fig. 6C). This result is in good agreement with previous studies indicating that RhoA/ROCK induces MMP activation [34, 35], and that the over-expression of Rho activates the EGFR via a MMP-dependent mechanism [36,37].

To investigate the mechanism of up-regulation of Egr-1 by AVP we examined the role of additional intracellular signalling proteins. We found that the c-Src inhibitor PP1 selectively blocked the AVP-induced expression of Egr-1, without any effect on the expression of c-Fos (Fig 7A). Similarly, A-10 cells transfected with the c-Src dominant negative mutant (SrcK295R/Y527F) [38] failed to trigger AVP-dependant Egr-1 up regulation (Fig 7B), whereas cells transfected with the wild-type c-Src responded as the untransfected cells. Moreover, A10 cells, transfected with a cDNA encoding a dominant negative mutant of Ras (L61S186Ras) [39] or treated with the MEK inhibitor PD98059, failed to elicit AVP- induced expression of Egr-1 without affecting the expression of c-Fos (Fig 7C and 7D).

We further explored the role of G protein signalling by blocking the coupling of G_i proteins to GPCRs via Pertussis toxin-catalyzed the ADP ribosylation of $G_{i\alpha}$, and by blocking the G protein $\beta\gamma$ -dependant signalling pathways by overexpression of CT- β GRK2 [40]. We found that A-10 cells pre-treated with Pertussis toxin or cells over-expressing the Pertussis toxin catalytic subunit did not affect the AVP-induced expression of c-Fos or Egr-1 (Fig. 8A, B). These findings agree with the coupling of V_1 receptors to Pertussis toxin insensitive G proteins [41]. Similarly, we found that cells over-expressing CT-GRK2 also failed to block the AVP-induced expression of both genes (Fig. 8C). Interestingly, we showed that AVP induced the formation of V_1 receptor/EGFR complexes as shown by expression of the V_1 receptor fused

to GFP in A10 cells, and co-immunoprecipitation of V₁ receptor fused to GFP and the endogenous EGFR (Fig. 9).

4. Discussion

We demonstrated that activation of the V₁ receptor up-regulated the expression of proliferation related genes, including c-Fos, Egr-1 and Cyclin D1, and increased the phosphorylation of the Rb protein. Further, we found that AVP-induced up-regulation of c-Fos and Egr-1 genes were mediated by the cross-talk of V₁ receptor and the EGFR signalling systems, which is in good agreement with previous studies indicating that AVP-stimulated cell proliferation via transactivation of the EGFR [11,42,43]. We also showed that AVP-up-regulated c-Fos and Egr-1 required the activation of PKC, the release of intracellular calcium and β -arrestin 2. These results are consistent with previous experiments indicating that V₁ receptor mutants lacking PKC phosphorylation sites failed to mediate DNA synthesis and progression through the cell cycle [44]. Most importantly, we showed that AVP-up-regulated c-Fos and Egr-1 by activation of two distinct EGFR transactivation signalling pathways. The expression of c-Fos was mediated by metalloproteinase-released HB-EGF; whereas the expression of Egr-1 was mediated by an AVP-activated a non-receptor tyrosine kinase, c-Src. It is likely that these two modes of EGFR transactivations are elicited by the differential phosphorylation of the EGFR. Indeed, the c-Src inhibitor PP1, inhibited the AVP-induced phosphorylation EGFR at residue 845, whereas the metalloprotease inhibitor GM6001 did not affect the phosphorylation of EGFR at that residue (unpublished results). Similar mechanisms of EGFR transactivation have been previously reported with other GPCR systems [27,45–49]. On the basis of our co-immunoprecipitation studies we argue that V_{1a} mediated EGFR transactivation involves the formation of V_{1a}/EGFR complex assembled with β -arrestin 2 and intracellular proteins of the trafficking mechanisms [50].

Interestingly, AVP-induced up-regulation of Egr-1, but not the c-Fos up-regulation, was mediated by a Ras/MEK-dependent signalling pathway. These results are in agreement with previous studies indicating that the GPCR-dependent expression of Egr-1 is via the activation of MEK/ERK signalling pathway [50–55]. Although ERK activation enhances transcription of c-Fos and Egr-1 [53,56–59], we found that AVP-induced expression of c-Fos is via an ERK-independent signalling pathway, indicating that c-Fos transcription is activated via multiple signalling pathways. Our data are consistent with a signalling model in which the activated and phosphorylated V₁ receptor binds β -arrestin to trigger the activation of Rho/ROCK- and c-Src-dependent signalling pathways. The Rho/ROCK pathway activates MMP to release HB-EGF, which then triggers the expression of c-Fos via the activation of the EGF receptor. On the other hand, the c-Src pathway catalyzes the phosphorylation of the EGFR, which elicit the activation of the Ras/MEK/ERK signalling mechanism to induce the expression of Egr-1 (Fig. 10). The novel mechanism of the AVP-triggered transactivation of EGFR provides the basis for regulating the selective expression of EIGs, as Egr-1 is a major vascular transcription factor involved in atherosclerosis and restenosis [60,61].

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Figure 1. Time-course of retinoblastoma protein (Rb) phosphorylation and protein expression of early immediate genes (c-Fos and Egr-1) and cyclin D 1 after AVP stimulation
A10 cells were incubated with 100 nM AVP for different time duration and cell extract subjected to Western blotting using specific anti-phosphorylated Rb (P-Rb), anti-c-Fos, anti-Egr-1 or anti-cyclin D1 and anti-actin which was used as a house keeping gene.

Figure 2. Time-course of transcriptional up-regulation of IEG after AVP or EGF stimulation of A-10 cells

A-10 cells were incubated with 100 nM AVP (A) or 1 nM EGF (B) for the different time duration; total RNA was prepared and subjected to semi-quantitative RT-PCR using specific primers for c-Fos, Egr-1 and GAPDH which was used as a house keeping gene. Data represent the mean $\pm$ SE of at least three experiments.

Figure 3. The transcriptional up-regulation of IEG is mediated the V_{1a} vasopressin receptor
A-10 cells were incubated with 1 μ M of the V_{1a} selective antagonist prior to the stimulation with 100 nM AVP. Total RNA was subjected to RT-PCR and products separated by agarose electrophoresis. Figures are representative of at least three independent experiments.

Figure 4. PKC, intracellular Ca^{+2} and β -arrestin 2 are involved in the transcriptional up-regulation of IEGs by transactivation of EGFR

A-10 cells were treated for 16 h. with PMA (A) to down-regulate PKC or with the specific inhibitor of PKC; Gö6983 (B) or with 10 or 50 μM BAPTA-AM (C) for 30 min prior to the AVP stimulation. A-10 cells were also transfected with a control siRNA (ct) or a siRNA for β -arrestin 2 (Arr) and then stimulated with 100 nM AVP. Then total RNA was submitted to RT-PCR and products separated by agarose electrophoresis. The down-regulation of PKCs by PMA completely inhibited the AVP-induced up-regulation of c-Fos and Egr-1 as well as Gö6983 and BAPTA at the highest dose (10 and 50 μM , respectively). The β -arrestin 2

silencing totally blocked the AVP-induced up-regulation of both genes. Figures are representative of at least three independent experiments.

Figure 5. The AVP-induced up-regulation of the IEGs is due to the transactivation of the EGFR A-10 cells were treated with 1 μ M of the EGFR tyrosine kinase inhibitor (A) or transfected with the dominant negative mutant HERCD533 (2, 4 and 6 μ g of DNA) (B) prior to the stimulation with 100 nM AVP. Total RNA was subjected to RT-PCR and products separated by agarose electrophoresis. Figures are representative of at least three independent experiments.

Figure 6. AVP-induced EGFR transactivation and transcriptional up-regulation of c-Fos is dependent upon the metalloproteinase and Rho kinase activities

A-10 cells were treated by two different metalloproteinase inhibitor; MMP inhibitor II (A) and GM6001 (B) or with a Rho kinase inhibitor (Y27632) (C) and then stimulated with 100 nM AVP. RT-PCR showed that both metalloproteinase and the Rho kinase inhibitors completely blocked the transcriptional up-regulation of c-Fos. Figures are representative of at least three independent experiments.

Figure 7. AVP-induced EGFR transactivation and transcriptional up-regulation of Egr-1 is dependent of the c-Src kinase activity and RAS/MEK pathway

A-10 cells were incubated with the c-Src inhibitor PP1 (A) or transfected with a c-Src dominant negative mutant (B) or with the dominant negative L61S186Ras (C) or incubated with the MEK inhibitor PD98059 (D) and then stimulated with AVP. RT-PCR showed that the inhibition of c-Src blocked the AVP-induced up-regulation of Egr-1 but it has no effect on that of c-Fos (A, B)). Similarly, the inhibition of Ras or MEK activities completely abrogated the Egr-1 up-regulation (C, D). Figures are representative of at least three independent experiments.

Figure 8. Neither pertussis toxin nor CT- β GRK2 has any affect on the AVP-mediated IEG up-regulation

A-10 cells were treated with Pertussis toxin (A) or transfected with plasmids encoding the catalytic subunit S1 (PTX S1) of the Pertussis toxin (B) or the c-terminus of the β -GRK2 (C). RT-PCR showed that neither Pertussis toxin nor the over-expression of S1 subunit or CT- β -GRK2 blocked the expression of these genes. Figures are representative of three independent experiments.

Figure 9. AVP-induced association of the V_{1a} vasopressin receptor and the EGFR

A-10 cells were transfected with the GFP-tagged V_{1a} vasopressin receptor and then were or not stimulated with 100 nM AVP. Cellular extracts were immunoprecipitated with anti-GFP/Protein A-agarose and subjected to Western blotting using anti-EGFR. After activation of the vasopressin receptor, a strong band, corresponding to the EGFR, can be seen.

Figure 10. Diagrammatic representation of the pathways involved in the IEG transcriptional up-regulation by the AVP-mediated EGFR transactivation