Arginine-Vasopressin Activates the JAK-STAT Pathway in Vascular Smooth Muscle Cells*

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JAK (Janus-activated kinase)-STAT (signal transducers and activators of transcription) signaling is a major signal transduction pathway in mammalian cells. Different growth factors and cytokines were reported as activators of the JAK-STAT pathway in various cell types. Interestingly, arginine-vasopressin (AVP) was never reported as an inducer of the JAK-STAT pathway. In the present study, we show for the first time that AVP stimulation of vascular smooth muscle cells (VSMCs) induces STAT3 tyrosine and serine phosphorylation, followed by nuclear translocation of the phosphorylated STAT3. In addition, we found that AVP induced JAK2 tyrosine phosphorylation. Taken together, these results demonstrate that AVP activates the JAK-STAT pathway in VSMCs. Furthermore, our results indicate that AVP-induced STAT3 tyrosine phosphorylation requires both JAK2 and c-Src tyrosine kinases. The present study also implicates that extracellular signal-regulated kinase (ERK1/2), which are serine/threonine kinases, are the mediators of STAT3 serine phosphorylation upon AVP stimulation. We further suggest that AVP-induced STAT3 serine phosphorylation negatively modulates AVP-induced STAT3 tyrosine phosphorylation. Finally, our results implicate a novel role for the JAK-STAT pathway, mediating AVP-induced VSMC hypertrophy.

The JAK3 (Janus-activated kinase)-STAT (signal transducers and activators of transcription) pathway is a major signal transduction pathway in mammalian cells. The JAK proteins belong to a family of tyrosine kinases, that consists of four family members, JAK1, JAK2, JAK3, and TYK2, with molecular mass of 120–130 kDa (1–4). STAT proteins are latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation mediated by JAK. STAT tyrosine phosphorylation promotes STAT homo- and heterodimerization via their Src homology 2 (SH2) domains, followed by translocation of activated STAT dimers to the nucleus. In the nucleus these proteins function as transcription factors, regulating the transcription, and therefore the expression, of various genes that participate in cell proliferation (1, 2, 4). Seven STAT proteins are known: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5B, and STAT6, with molecular masses of 80–95 kDa (1–4).

As in other cell types, the JAK-STAT pathway has a major role in VSMCs. The abundant isoforms in VSMCs are STAT1, STAT3, and JAK2. The JAK-STAT pathway could be activated in VSMCs through both tyrosine kinase receptor (YKRs) (5, 6) and G protein-coupled receptors (GPCRs) (7, 8). For instance, epidermal growth factor (EGF), platelet derived growth factor (PDGF), thrombin, and angiotensin II (AngII) are all known activators of the JAK-STAT pathway in VSMCs (5–8). Almost all hormones and growth factors that activate the JAK-STAT pathway in VSMCs will eventually lead to cell proliferation (1–4).

YKRs, cytokine receptors and GPCRs are all capable of activating the JAK-STAT pathway. In the case of JAK-STAT activation through YKRs (like EGF and PDGF) the tyrosine kinase receptor undergoes autotyrosine phosphorylation thus providing docking sites for both JAK and STAT proteins, which bind to the receptor via their SH2 domain. Once associated to the receptor, the STAT proteins are phosphorylated on conserved tyrosine residues by JAK (2, 9). Unlike YKRs, GPCRs lack the cytoplasmic tyrosine phosphorylation sites. Therefore, the mechanism of GPCRs-induced activation of the JAK-STAT pathway appears to be more elusive and intricate. It was previously described that GPCRs-induced tyrosine phosphorylation and activation of STAT proteins appears to require the JAK family of tyrosine kinases (10). In addition, the Src family of tyrosine kinases has also been implicated in phosphorylation and activation of STAT proteins following activation of GPCRs (11). Furthermore, previous reports have shown that GPCR-induced tyrosine phosphorylation of different STAT isoforms seems to require different JAK and Src isoforms (7, 10).

Besides the tyrosine phosphorylation, which is required for its activation and dimerization, STAT3 contains a single serine phosphorylation site, serine 727, which is located within a Pro-Met-Ser-Val-Phe (PMSP) MAPK recognition sequence (12). Therefore, it is not surprising that most reports indicate that upon stimulation with various growth factors, serine phosphorylation of STAT3 is mediated mainly by the MAPK proteins, and especially by extracellular signal regulated kinase 1/2 (ERK1/2) (12, 13). Serine phosphorylation of STAT3 is required for maximal transcriptional activity of the activated STAT dimer and appears to negatively modulate the tyrosine phosphorylation of STAT3 (13).

Arginine vasopressin (AVP) is a vasoactive peptide, which leads to VSMC hypertrophy through activation of GPCR (14–16). Interestingly, AVP was never reported as an activator of the JAK-STAT pathway. In the present study we show for the first time that AVP signaling through its GPCR activates the JAK-STAT pathway in VSMCs. We also examined whether JAK2 and c-Src are required for AVP-induced tyrosine phosphorylation of STAT3 in VSMCs. Considering the major role of STAT3 serine phosphorylation in regulating STAT3 activity, we attempted to identify the serine/threonine kinases that are responsible for AVP-induced STAT3 serine phosphorylation. We also examined the cross-talk between STAT3 serine phosphorylation and STAT3 tyrosine phosphorylation upon AVP stimulation of VSMCs. Finally, we

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3 The abbreviations used are: JAK, Janus-activated kinase; STAT, signal transducers and activators of transcription; VSMC, vascular smooth muscle cell; AVP, arginine-vasopressin; ERK1/2, extracellular signal-regulated kinase 1/2; SH2, Src homology 2; YKR, tyrosine kinase receptor; GPCR, G protein-coupled receptor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; AngII, angiotensin II; PMSP, Pro-Met-Ser-Pro; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.
investigated a possible involvement of the JAK-STAT pathway in AVP-induced hypertrophy.

EXPERIMENTAL PROCEDURES

Materials—The control (scrambled) oligonucleotides, the p60 c-Src antisense oligonucleotides and the anti-total STAT3, JAK2, and actin antibodies were purchased from Santa Cruz Biotechnology, and Lipofectin was purchased from Invitrogen. AG490, SB203580, and PD98059 were purchased from Calbiochem, and phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Total ERK2 antibody was prepared as previously described (17). Goat anti-rabbit antibody (horseradish peroxidase-conjugated) was purchased from Pierce, and goat antimouse antibody (horseradish peroxidase-conjugated), phosphospecific anti-ERK1/2 antibody and Hoechst 33258 were purchased from Sigma. Phosphotyrosine-specific and phosphoserine-specific anti-STAT3 antibodies were purchased from Calbiochem. Phosphotyrosine-specific anti-JAK2 antibody was purchased from Upstate, and anti-c-Src antibody was purchased from Santa Cruz Biotechnology.

Cell Culture—Primary rat VSMCs were isolated and cultured in 60-mm culture dishes in minimum essential medium, supplemented with 10% fetal calf serum, 2% l-glutamine, and 1% penicillin-streptomycin antibiotics as described previously (18, 19). For all experiments, confluent VSMCs from the second through seventh passages were used. Cells were incubated with fetal calf serum-free minimum essential medium for 18–24 h prior to stimulation with different growth factors.

Preparation of VSMC Lysates—VSMCs were treated with the various modulators and growth factors for the indicated times, the reaction was stopped with liquid nitrogen, and then the cells were lysed in 500 μl of lysis buffer (25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 0.5% deoxycholic acid, 10% glycerol, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). Cells were scraped from the plates and gently sonicated (2 × 5 s, 40 W, 4 °C) and clarified by centrifugation at 17,000 × g for 20 min at 4 °C. Protein concentration in cell extracts was determined according to Bradford analysis (20).

Cellular Fractionation—The preparation of nuclear and cytosolic fractions was carried out as described previously (21). Briefly, after stimulation with AVP (100 nM), the cells (3 × 10^6) were washed, scraped into 0.5 ml of buffer A (50 mM β-glycerophosphate, pH 7.3, 1.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM Na3VO4, supplemented with 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), centrifuged (12,000 × g, 5 min, 4 °C), and resuspended in 200 μl of the lysis buffer supplemented with 0.1% Nonidet P-40. The lysate was mixed vigorously and centrifuged as above to yield a supernatant that contained the cytosol fraction. Nuclear proteins were extracted by resuspending the nuclear pellet in 100 μl of extraction buffer (420 mM NaCl, 50 mM β-glycerophosphate, 0.5 mM Na3VO4, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 25% glycerol), brief sonication (2 × 5 s, 40 W, 4 °C), vigorous mixing, and centrifugation.

Western Blotting—After separation of extracts by 10% SDS-PAGE, proteins were immunoblotted using total ERK2, total STAT3, phosphospecific anti-ERK1/2, phosphotyrosine-specific anti-JAK2, anti-c-Src antibody, and phosphotyrosine-specific and phosphoserine-specific anti-STAT3 antibodies. In addition, protein levels were normalized against total ERK protein levels. Immunoreactive protein bands were visualized using ECL (Amersham Biosciences) Western blotting detection system.

Oligonucleotide Treatment—Oligonucleotide treatment was carried out using Lipofectin as described previously by Duff et al. (22). After treatment with either the control (scrambled) or the c-Src antisense oligonucleotides for 24 h, the medium was removed, Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum was added, and the cells were allowed to recover for 30 min before stimulation with AVP.

Protein/DNA Ratio—VSMCs were either pretreated with AG490 or received no treatment prior to stimulation with AVP for 2, 24, or 48 h. Then the DNA content and protein content of the cells were determined. The DNA content of the cells was determined using the Hoechst 33258 assay (23). The cells were transferred from the culture dishes to a 96-well plate in 100 μl of fetal calf serum-free minimum essential medium and then 100 μl of Hochecht solution (10 μl/ml) was added to the well. Following a 10-min incubation, the absorbance was read on a microtiter plate spectrophotometer at 340 nm. For protein content determination, cells were lysed, and the protein concentration in cell extracts was determined according to Bradford analysis (20).

Statistical Analysis—The protein/DNA ratio experiments were carried out three separate times, and each experiment contained triplicates of the various treatments. The significance of the results was determined by two-way analysis of variance (p < 0.05), performed by STATISTICA software.

RESULTS

AVP Activates STAT3 and JAK2 in VSMCs—Currently, it has not been reported that AVP is capable of activating the JAK-STAT pathway. To examine whether AVP induces tyrosine phosphorylation of STAT3 in cultured rat aortic VSMCs, growth-arrested cells were treated for 10 min with AVP (100 nM). Other treatments were with AngII (100 nM for 10 min) and with EGF (1 nM for 10 min), both are known activators of the JAK-STAT pathway in VSMCs. Cells were lysed and immunoblotted with anti-STAT3 phosphotyrosine-specific antibody that recognizes the tyrosine 705-phosphorylated (but not the non-phosphorylated) form of STAT3. As shown in Fig. 1, AVP induced a significant tyrosine phosphorylation of STAT3, compared with untreated cells (negative control), indicating that AVP activates STAT3 in VSMCs.

To determine the time dependence of AVP-induced STAT3 tyrosine phosphorylation, growth-arrested cells were treated at different times with AVP (100 nM). Cell lysates were subjected to immunoblot using anti-STAT3 phosphotyrosine-specific antibody (Fig. 2A), and densitometric analysis of the results was carried out (Fig. 2B). As shown in Fig. 2B, AVP induces STAT3 tyrosine phosphorylation in a biphasic manner, with peaks following 15 and 60 min of stimulation with AVP. In addition, STAT3 serine phosphorylation was also examined using an anti-phosphoserine STAT3 antibody. Treatment with AVP led to a monophasic increase in STAT3 serine phosphorylation, with the highest level observed at 5–15 min (data not shown).
Because several studies have demonstrated JAK2 involvement in STAT tyrosine phosphorylation in response to other growth factors (8, 10), we examined whether AVP induces tyrosine phosphorylation and thus activation of JAK2. Therefore, we examined JAK2 tyrosine phosphorylation in response to stimulation with AVP for different time periods. Tyrosine phosphorylation of JAK2 was detected by immunoblotting cell lysates with phosphotyrosine-specific anti-JAK2 antibody (Fig. 2A). As shown in Fig. 2B, a densitometric analysis shows a biphasic increase in JAK2 tyrosine phosphorylation following treatment with AVP, with maximal activation of JAK2 following 10 and 60 min. Of note, JAK2 tyrosine phosphorylation, and thus activation, precedes STAT3 tyrosine phosphorylation, suggesting a possible role for JAK2 as the mediator of AVP-induced STAT3 tyrosine phosphorylation.

Phosphorylation of STAT proteins, including STAT3, promotes their translocation to the cell nucleus, where they induce the transcription of their target genes. To determine whether AVP-induced STAT3 tyrosine phosphorylation was accompanied by nuclear translocation, nuclear and cytosolic fractions of AVP-treated VSMCs were obtained as described under “Experimental Procedures” and immunoblotted with anti-total STAT3 and phosphotyrosine-specific anti-STAT3 antibodies. As shown in Fig. 3, AVP induced not only tyrosine phosphorylation of STAT3 but also nuclear translocation of the phosphorylated STAT3 proteins.

JAK2 and c-Src Tyrosine Kinases Are Involved in AVP-induced STAT3 Tyrosine Phosphorylation—JAK2 was previously reported as the mediator of AVP-induced STAT3 tyrosine phosphorylation in response to various growth factors. To test whether AVP-induced STAT3 tyrosine phosphorylation is also mediated by JAK2, we used AG490, a selective JAK2 inhibitor, which inhibits JAK2 at low micromolar concentrations (<5 μM) but does not inhibit the family of Src tyrosine kinases even at higher concentrations than 50 μM (24). Cells were pretreated with AG490 (10 μM) for 1 h prior to stimulation with AVP (100 nM) for 0, 15, and 60 min. As shown in Fig. 4A, AG490 inhibited AVP-induced JAK2 tyrosine phosphorylation. Furthermore, AVP-induced STAT3 tyrosine phosphorylation was greatly diminished by pretreatment with AG490, compared with the untreated cells (Fig. 4B). Therefore, we suggest that AVP-induced STAT3 tyrosine phosphorylation is dependent on the activity of JAK2 and probably involves direct JAK2 phosphorylation of STAT3 on tyrosine residues. Interestingly, STAT3 serine phosphorylation was not altered upon pretreatment with AG490 (Fig. 4C).

STAT3 activation via GPCRs, for instance upon AngII and thrombin stimulation, appears to be mediated via different members of the Src family of tyrosine kinases. To determine whether p60 c-Src (c-Src) is involved in AVP-induced STAT3 tyrosine phosphorylation in VSMCs, we carried out experiments in which we utilized a specific c-Src antisense oligonucleotide.
gonucleotide to suppress c-Src protein expression. VSMCs were treated with a control (scrambled) oligonucleotide or with the c-Src antisense oligonucleotide using Lipofectin, for 24 h prior to stimulation with AVP (100 nM) for 60 min. Expectedly, treatment with the c-Src antisense oligonucleotide greatly diminished c-Src protein expression compared with treatment with the control (scrambled) oligonucleotide (Fig. 5A). As shown in Fig. 5B, the c-Src antisense oligonucleotide treatment significantly abrogated AVP-induced STAT3 tyrosine phosphorylation, suggesting that c-Src is required for AVP-induced STAT3 tyrosine phosphorylation.

**AVP-induced STAT3 Serine Phosphorylation Is Mediated by ERK1/2**—STAT3 serine phosphorylation has a major role in enhancing STAT3 transcriptional activity. Serine 727, which is the single serine phosphorylation site in STAT3, is located within a PMSP MAPK recognition sequence. Accordingly, previous studies have shown that upon stimulation with various growth factors, such as AngII, EGF, and PDGF, serine phosphorylation of STAT3 is mediated by the MAPK family members, and especially by ERK1/2 (12, 13).

Interestingly, it was demonstrated that AVP induces ERK1/2 phosphorylation and activation in VSMCs (18). To find whether STAT3 serine phosphorylation upon AVP stimulation is mediated through the MAPK pathway, and specifically by ERK1/2, we used the specific MEK inhibitor, PD98059 (25). Cells were pretreated with PD98059 (20 μM) for 20 min prior to stimulation with AVP (100 nM) for 0, 15, and 60 min. Pretreatment with the MEK inhibitor, PD98059 significantly blocked the phosphorylation and therefore the activation of ERK1/2, the downstream targets of MEK (Fig. 6A). As shown in Fig. 6B, PD98059 also led to a decrease in STAT3 serine phosphorylation, suggesting that AVP-induced STAT3 serine phosphorylation is mediated by ERK1/2. Furthermore, treatment with SB203580 (10 μM, for 30 min prior to AVP stimulation), which is a specific p38 MAPK inhibitor (26), had no significant effect on STAT3 serine phosphorylation indicating that p38 MAPK is not responsible for AVP-induced STAT3 serine phosphorylation (Fig. 6E).

**AVP-induced STAT3 Serine Phosphorylation Negatively Modulates Its Tyrosine Phosphorylation**—STAT3 serine phosphorylation was previously reported to negatively modulate STAT3 tyrosine phosphorylation (13). To find whether STAT3 serine phosphorylation negatively modulates STAT3 tyrosine phosphorylation upon stimulation with AVP, we also examined the effect of PD98059 pretreatment on AVP-induced STAT3 tyrosine phosphorylation. As shown in Fig. 6C, PD98059, which inhibited AVP-induced STAT3 serine phosphorylation, induced a significant elevation in AVP-induced STAT3 tyrosine phosphorylation, implies that upon AVP stimulation STAT3 serine phosphorylation negatively modulates STAT3 tyrosine phosphorylation.

To confirm these results we carried out experiments using PMA. PMA is a known modulator of protein kinase C (PKC) and subsequently of the MAPK pathway in various cell types (16, 27–30). In VSMCs, PKCa is the most abundant PKC isof orm (31). Previous studies and our results (data not shown) have demonstrated that a 24-h exposure to

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**FIGURE 5.** The effect of c-Src antisense oligonucleotide on AVP-induced STAT3 tyrosine phosphorylation in VSMCs. VSMCs were treated for the times shown with AVP (100 nM) following either pretreatment with control (scrambled) oligonucleotide alone or with c-Src antisense oligonucleotide. Cells were lysed, and lysates were immunoblotted with anti-c-Src antibody (A), phosphotyrosine-specific anti-STAT3 antibody (B), and anti-total STAT3 antibody (C). Results shown are representative of different independent experiments (n = 2).

**FIGURE 6.** PD98059 modulates AVP-induced STAT3 tyrosine and serine phosphorylation in VSMCs. Growth-arrested VSMCs were incubated in the presence or absence of PD98059 (20 μM) for 30 min and then treated with AVP (100 nM) for the indicated times. Cells were then lysed, and lysates were subjected to Western immunoblot using phosphospecific anti-ERK1/2 antibody (A), phospho-tyrosine-specific anti-STAT3 antibody (B), and anti-total STAT3, ERK, and actin antibodies (D). Similar results were obtained in separate experiments (n = 3).

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PMA leads to a significant down-regulation of PKCα in several cell types, including VSMCs. Because the MAPK pathway is known to be activated via PKCα in VSMCs upon activation of GPCR (32), we investigated the involvement of PKCα in the MAPK pathway in response to AVP stimulation. Therefore, cells were either pretreated with PMA for 24 h or received no pretreatment, before stimulation with AVP (100 nM) for 0, 15, and 60 min. As expected, PMA treatment for 24 h markedly inhibited AVP-induced phosphorylation of ERK1/2 (Fig. 7A).

The abrogated phosphorylation of ERK1/2, because of the 24-h exposure to PMA, led to a significant decrease in AVP-induced STAT3 serine phosphorylation (Fig. 7B), further indicating that STAT3 serine phosphorylation in response to AVP is mediated by ERK1/2. Furthermore, the 24-h exposure to PMA, which led to decreased STAT3 serine phosphorylation, also led to a significant increase in AVP-induced STAT3 tyrosine phosphorylation, compared with cells that were not pretreated with PMA prior to AVP stimulation (Fig. 7C). These results support the notion that the negative modulation of STAT3 serine phosphorylation on STAT3 tyrosine phosphorylation occurs upon stimulation of VSMCs with AVP.

Short term exposure to PMA (10 min) activates PKCα and subsequently the MAPK pathway (16, 27–30). To verify the results regarding STAT3 serine phosphorylation, cells were pretreated with PMA (2.5 μM) for 10 min prior to stimulation with AVP (100 nM) for 0, 15, or 60 min. As expected, pretreatment with PMA for 10 min markedly enhanced ERK1/2 phosphorylation in response to AVP (Fig. 8A). Accordingly, AVP-induced STAT3 serine phosphorylation was also augmented in PMA-treated cells (Fig. 8B), confirming that STAT3 serine phosphorylation in response to AVP stimulation is directly mediated by ERK1/2. As shown in Fig. 8C, pretreatment with PMA for 10 min significantly decreased STAT3 tyrosine phosphorylation in response to AVP. These results provide yet another indication that AVP-induced serine phosphorylation of STAT3 negatively regulates its tyrosine phosphorylation.

Next, we attempted to elucidate the mechanism by which STAT3 serine phosphorylation negatively modulates its tyrosine phosphorylation upon AVP stimulation. Therefore, we examined whether JAK2 is involved in that process. As shown in Fig. 8D, PMA treatment for 10 min prior to AVP stimulation, which enhanced AVP-induced STAT3 serine phosphorylation and reduced AVP-induced STAT3 tyrosine phosphorylation, also led to a significant decrease in AVP-induced JAK2 tyrosine phosphorylation. Furthermore, PMA treatment for 24 h prior to AVP stimulation, which abrogated AVP-induced STAT3 serine phosphorylation and enhanced AVP-induced STAT3 tyrosine phosphorylation, also led to a significant increase in AVP-induced JAK2 tyrosine phosphorylation (Fig. 7D). These results strongly suggest that JAK2 is involved in the mechanism by which STAT3 serine phosphorylation negatively regulates STAT3 tyrosine phosphorylation upon AVP stimulation.

The JAK-STAT Pathway Is Involved in AVP-induced VSMC Hypertrophy—Following our discovery that AVP activates the JAK-STAT pathway in VSMCs, it was interesting to find out the physiological consequences of that activation. As mentioned before, AVP leads to VSMC hypertrophy (14–16), whereas the JAK-STAT pathway was only implicated in VSMC proliferation and migration by various growth factors. Therefore, it was interesting to examine whether the JAK-STAT pathway is also involved in AVP-induced VSMC hypertrophy. Therefore, VSMCs were either treated with AG490 for 1 h or received no treatment prior to AVP treatment for 24 or 48 h. Hypertrophy is an increase in cell size because of elevation in protein content without DNA replication. Therefore, the hypertrophy assay was carried out by measuring the ratio between the protein content and the DNA content of the treated VSMCs. The protein content was measured by the Bradford method (20), and the DNA content was measured.
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![Image](60x381 to 288x733)

FIGURE 8. Short exposure to PMA modulates AVP-induced STAT3 tyrosine and serine phosphorylation in VSMCs. Growth-arrested VSMCs were incubated in the presence or absence of PMA (2.5 μM) for 10 min and then treated with AVP (100 nM) for the indicated times. Cells were then lysed, and lysates were immunoblotted with phospho-specific anti-ERK1/2 antibody (A), phosphoserine-specific anti-STAT3 antibody (B), phosphotyrosine-specific anti-STAT3 antibody (C), phosphotyrosine-specific anti-JAK2 antibody (D), and anti-total STAT3, ERK, JAK2, and actin antibodies (E). Similar results were obtained in separate experiments (n = 3).

AVP (time, min) 0 15 15 60 60
FMA (2.5μM, 10min) - + + + +

using the Hoechst 33258 reagent, a fluorescent dye, which binds to the DNA of the cells (see "Experimental Procedures" and Ref. 23). Then, the ratio between the protein content and the DNA content is calculated for each treatment and is normalized according to the control, untreated cells. As shown in Fig. 9, treatment with AVP alone or following incubation with AG490 did not significantly affect cell viability, as indicated by the unaltered DNA content. However, the AVP treatment led to a significant increase in protein content, an increase that is inhibited by AG490 pretreatment. Accordingly, AVP treatment for 24 and 48 h leads to VSMC hypertrophy, as indicated by the decrease in the protein/DNA ratio to control levels. These results suggest that the JAK-STAT pathway has a major role in AVP-induced VSMC hypertrophy.

DISCUSSION

As previously described the JAK-STAT pathway is activated by various cytokines and growth factors, such as PDGF, EGF, interleukin-6, and AngII (5–8, 33). However, AVP was never reported as an activator of the JAK-STAT pathway. The results of the current study show for the first time that AVP induces both tyrosine and serine phosphorylation of STAT3, followed by nuclear translocation of the phosphorylated STAT3. Our results also demonstrate that both JAK2 and c-Src are required for AVP-induced STAT3 tyrosine phosphorylation. In addition, serine phosphorylation of STAT3 upon AVP stimulation appears to be mediated directly by ERK1/2 and was also shown to negatively modulate STAT3 tyrosine phosphorylation. Finally, our results indicate that the JAK-STAT pathway has a major role in AVP-induced VSMC hypertrophy.

GPCR-induced tyrosine phosphorylation of STAT proteins often requires the involvement of the JAK and Src tyrosine kinases. In addition, different STAT isofoms requires different members of the JAK and Src families upon stimulation with different growth factors. For instance, both JAK2 and the Src family p59 Fyn tyrosine kinases are required for AngII-induced STAT1 tyrosine phosphorylation, whereas c-Src is required for AngII-induced STAT3 tyrosine phosphorylation (7, 10). JAK2 was also shown to be a prerequisite for both STAT1 and STAT3 tyrosine phosphorylation and nuclear translocation upon thrombin stimulation of VSMCs (8). In the present study we showed that AVP induces JAK2 phosphorylation and that inhibiton of JAK2 with AG490 inhibits AVP-induced STAT3 tyrosine phosphorylation. In addition, down-regulation of c-Src using c-Src-specific antisense oligonucleotide abolishes AVP-induced STAT3 tyrosine phosphorylation. Thus, our results indicated that both JAK2 and c-Src are required for AVP-induced STAT3 tyrosine phosphorylation.

Ram et al. (11) previously suggested that Src proteins may serve as scaffolds for both STAT and JAK proteins, thus mediating GPCR-induced activation of the JAK-STAT pathway. Src activation was previously demonstrated upon AVP stimulation of various cell types (34, 35). Considering these studies and the results presented in this study, we propose that AVP binding to its GPCR activates both c-Src and JAK2, thereby inducing the formation of a c-Src-JAK2 complex. The activated c-Src may serve as a docking site for STAT3, which is then phosphorylated on tyrosine residues by JAK2, STAT3 was previously reported as a substrate for c-Src in vitro, and Liang et al. (7) even suggested that c-Src directly phosphorylates STAT3 upon AngII stimulation of VSMCs. On the other hand, JAK2 was reported to phosphorylate STAT3 in vivo on tyrosine residues upon stimulation with several growth factors and cytokines. In addition, our results showed that AVP induces JAK2 tyrosine phosphorylation and activation prior to STAT3 tyrosine phosphorylation and that phosphorylated and activated JAK2 is required for AVP-induced STAT3 tyrosine phosphorylation. Therefore, we predict that upon AVP stimulation, STAT3 is tyrosine-phosphorylated by JAK2, whereas c-Src serves as a scaffold for both JAK2 and STAT3. However, further study is required to find whether phosphorylated and active c-Src is required and to elucidate the mechanism by which AVP induces STAT3 tyrosine phosphorylation.

Importantly, the present study show that AVP not only induces STAT3 tyrosine phosphorylation but also leads to nuclear translocation of the phosphorylated STAT3 proteins. Of note, tyrosine-phosphorylated STAT3 was undetectable in the cytoplasmic fraction, probably because of the immediate translocation of the phosphorylated STAT3 into the nucleus upon AVP stimulation. In addition, after STAT3 completes its transcriptional role in the nucleus, it undergoes rapid dephosphorylation by specific phosphatases, causing the fraction of tyrosine-phosphorylated STAT3 in the cytoplasm to be extremely low and therefore undetectable.

Besides phosphorylation on tyrosine residues, STAT3 is also phosphorylated on serine residues in response to several cytokines and growth factors. As mentioned before, phosphorylation of STAT3 on Ser277 is required for maximal transcriptional activity, and it occurs...
within a PMSP MAPK recognition sequence. Therefore, it is not surprising that previous reports have indicated that STAT3 serine phosphorylation is mediated via the members of the MAPK family and mainly by ERK1/2 (12, 13). For example, EGF induces the serine phosphorylation of STAT3 by ERK1/2 (13). In addition, IL-2 and AngII-induced STAT3 serine phosphorylation was shown to be MEK/ERK-dependent (7, 36).

Our results demonstrate that AVP induces STAT3 serine phosphorylation in a monophasic manner, with high levels of serine phosphorylated STAT3 observed at 5–15 min following AVP stimulation. Interestingly, the mode of AVP-induced STAT3 serine phosphorylation corresponds to that of the transient AVP-induced ERK1/2 phosphorylation and activation (18). In addition, AVP-induced STAT3 serine phosphorylation was blocked upon treatment with PD98059, which is a specific inhibitor for MEK and consequently for ERK1/2. In contrast, the specific p38 inhibitor, SB203580, did not affect AVP-induced STAT3 serine phosphorylation, demonstrating that p38 is not involved in AVP-induced STAT3 serine phosphorylation. Long exposure to PMA, which causes the down-regulation of PKCz and abrogation of ERK1/2 activation, also diminished AVP-induced STAT3 serine phosphorylation. Furthermore, enhanced activation of ERK1/2, because of short exposure to PMA, led to augmented STAT3 serine phosphorylation in response to AVP. Taken together, these results strongly suggest that AVP-induced STAT3 serine phosphorylation is directly mediated by ERK1/2. In addition, incubation of the cells with both PMA for 10 min and PD98059 prior to AVP stimulation did not lead to decreased tyrosine phosphorylation of STAT3 as compared with AVP treatment alone (data not shown), indicating that the PKC-dependent decrease in tyrosine phosphorylation of STAT3 (Fig. 8C) is also MEK-dependent. Of note, although the PKC pathway plays a major role in AVP-induced activation of the MAPK pathway, it is possible that other signaling pathways also lead to MAPK activation and STAT3 serine phosphorylation upon AVP stimulation.

Chung et al. (13) has previously suggested that STAT3 serine 727 phosphorylation negatively modulates its tyrosine phosphorylation. Also noteworthy, negative modulation was demonstrated upon EGF stimulation of COS cells that transiently expressed STAT3. For instance, it was demonstrated that EGF-induced tyrosine phosphorylation of the S727A STAT3 mutant was significantly higher than that of the wild type STAT3 protein expressed under similar condition in these cells. Our results showed that treatments with PD98059 and with PMA (for 24 h) that abolished AVP-induced STAT3 serine phosphorylation, caused by inhibition of ERK1/2, also enhanced AVP-induced STAT3 tyrosine phosphorylation. In addition, short exposure to PMA, which augmented AVP-induced STAT3 serine phosphorylation because of enhanced ERK1/2 activation, also decreased STAT3 tyrosine phospho-
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Tyrosine phosphorylation and not serine phosphorylation that negatively modulates the tyrosine phosphorylation by specific phosphatases. Also noteworthy, pretreatment with AG490, which inhibits AVP-induced tyrosine phosphorylation, had no effect on AVP-induced serine phosphorylation, indicating that it is the AG490, which inhibits AVP-induced tyrosine phosphorylation, also significantly enhances JAK2 tyrosine phosphorylation. Taken together, these results strongly suggest that AVP-induced STAT3 serine phosphorylation negatively regulates STAT3 tyrosine phosphorylation in a mechanism that involves JAK2. Therefore, a potential mechanism could be that phosphorylation of STAT3 on serine 727 modulates the accessibility of the tyrosine 705 residue to the rapid dephosphorylation upon AVP treatment. Taken together, these results indicate that the JAK-STAT pathway plays a major role in AVP-induced VSMC hypertrophy, possibly revealing a novel physiological consequence for the JAK-STAT pathway in VSMCs.

In conclusion, in the present study AVP was shown to activate the JAK-STAT pathway, an important signaling pathway that appears to be involved in AVP-induced hypertrophy. AVP induces both JAK2-dependent STAT3 tyrosine phosphorylation and ERK1/2-dependent serine phosphorylation. The intriguing AVP-induced cross-talk between the PKC-ERK pathway and the JAK-STAT pathway is demonstrated by the unique negative modulation of STAT3 serine phosphorylation on its tyrosine phosphorylation. The cross-talk between the serine phosphorylation and the tyrosine phosphorylation appears to be crucial for regulating STAT3 activity, and further research is required to elucidate the role of that cross-talk in STAT3 dimerization, nuclear translocation, and transcription.

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