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Linkage between α_1 Adrenergic Receptor and the Jak/STAT Signaling Pathway in Vascular Smooth Muscle Cells

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The Jak/STAT pathway is activated following stimulation of the type I angiotensin II receptor. To examine whether this pathway is shared among other G-protein-coupled receptors, we studied the linkage between the α_1 adrenergic receptor and this pathway. The α_1 agonist phenylephrine induced tyrosine phosphorylation of Jak2, Tyk2, and STAT1 in vascular smooth muscle cells. The phosphorylation of Jak2 was prevented by the α_1 receptor antagonists prazosin and chloroethylclonidine, but not by WB4101, and that of STAT1 was inhibited by prazosin and the Jak2 inhibitor AG490. After stimulation with phenylephrine, Jak2 and STAT1 were found to associate with α_{1B} receptor. Phenylephrine stimulated the DNA binding activity of STAT1. Protein synthesis promoted by phenylephrine was inhibited by prazosin, AG490, and the introduction of a decoy oligonucleotide for STAT1. These results suggested that α_1 receptor is linked to the Jak/STAT pathway and that this pathway mediates α_1 agonist-induced smooth muscle hypertrophy.

The Janus-activated kinase (Jak)/signal transducers and activators of transcription (STAT) pathway is an established signal transduction system for cytokines (1, 2). When a cytokine receptor is stimulated, Jak kinases associating with the cytosolic domain of the receptor are activated to phosphorylate receptor tyrosines. STATs bind to the receptor by recognizing phosphorylated receptor tyrosines with their SH2 domain. Thereafter, they are phosphorylated by Jaks. After dimerization, phosphorylated STATs translocate into nuclei to transactivate target genes.

It is also established that seven-transmembrane receptors for calcium-mobilizing vasoactive substances such as angiotensin II, endothelin-1, thrombin, and noradrenaline are linked to guanine nucleotide-binding protein (G-protein). Contraction of vascular smooth muscle cells (VSMCs) is triggered by the G-protein-dependent activation of phospholipase C, which generates two intracellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol.

In the meantime, several proteins are tyrosine-phosphorylated following stimulation with vasoconstrictors, particularly angiotensin II (3–5). More specifically, angiotensin II induces phosphorylation of Jaks and STATs through the type 1 angiotensin II receptor (AT_1) in VSMCs and cardiac myocytes (6–8). Collectively, angiotensin II activates Jak1, Jak2, Tyk2, STAT1, STAT2, and STAT3 in these cells (8–10). Recently STAT5 also has been shown to be activated by angiotensin II in cardiac myocytes (11). However it remains uncertain whether the Jak/STAT pathway is activated only by AT_1 receptor, or this is a common pathway for G-protein-coupled receptors. Cellular functions regulated by this pathway are also unknown.

Hypertension induces arterial wall thickening and cardioventricular hypertrophy, which is caused at least in part by the increase in the volume of VSMCs and cardiac myocytes. These remodeling processes are believed to be adaptive responses required to resist elevated blood pressure. Several *in vitro* and *in vivo* studies have suggested that angiotensin II plays an important role in the induction of hypertrophy in VSMCs and cardiac myocytes (12–16). Noradrenaline also has been suggested to induce hypertrophy of VSMCs and cardiac myocytes through α_1, in particular α_{1B}, receptor (17–19). It remains unclear, however, how these agonists transduce their signals for cellular hypertrophy.

Recently the Jak/STAT pathway has been suggested to play a key role in the development of cardiac myocyte hypertrophy induced by interleukin 6-related cytokines that signal through the transmembrane pro-

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tein gp130, such as cardiotrophin-1 and leukemia inhibitory factor (20–23). Therefore we were interested in whether this pathway mediates VSMC hypertrophy induced by vasoconstrictors. In this study, first we investigated whether α_1 adrenergic receptor agonist stimulates the Jak/STAT pathway and secondly whether this pathway is involved in α_1 agonist-induced VSMC hypertrophy.

METHODS

Chemicals. Phenyleprine, prazosin, WB4101, chloroethylclonidine (CEC), PD123319, and tyrphostin B42 (AG490) were purchased from Research Biochemicals International. Human angiotensin II and endothelin-1 were obtained from Peptide Institute. Human thrombin and cycloheximide (CHX) were from Sigma Chemical Co. Human interferon-γ was from PeproTech. Candesartan (CV-11974) was a gift from Takeda Chemical Industries.

Cell culture. VSMCs obtained from the media of human umbilical arteries by explant culture were used in the third passage. They were identified by immunostaining with anti-smooth muscle α-actin (Dako). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% (v/v) fetal bovine serum (FBS, Life Technologies), 5 ng/ml human recombinant basic fibroblast growth factor (bFGF, Amersham Pharmacia Biotech), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (growth medium). A quiescent state was achieved by incubating subconfluent cells in DMEM containing 0.1% bovine serum albumin and 0.4% FBS for 48 h. Cell numbers were determined using a Coulter counter (Z1).

Transfection of oligonucleotides. Double-stranded oligonucleotides were prepared by annealing sense and antisense phosphorothioate oligodeoxynucleotides of the consensus sequence for STAT1 (5′-CATGTTATGCATATTCCTGTAAGTG-3′). Mutant oligonucleotides were also synthesized (CATGTTATGCATATTGGAGTAAGTG-3′). The nucleotides were introduced into cells using FuGENE 6 transfection reagent (Boehringer-Mannheim), according to the manufacturer's protocol.

Protein and DNA synthesis. Protein and DNA synthesis were measured by the level of leucine and thymidine incorporation, respectively. Quiescent cells cultured in 24-well plates were labeled with 37 kBq/ml L-[4, 5-^{3}H]leucine (6.66 TBq/mmol, Amersham Pharmacia Biotech) or [6-^{3}H]thymidine (0.74–1.1 TBq/mmol, Amersham Pharmacia Biotech) for 24 h. The medium was discarded and the cells were washed three times with 0.5 ml of ice-cold phosphate-buffered saline containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The cells were precipitated with 0.5 ml of 5% trichloroacetic acid for 10 min, then the acid was removed with 0.5 ml of ethanol:diethyl ether (3:1, v/v). The precipitates were lysed with 0.5 ml of 0.3 M NaOH, neutralized with 2 M HCl, and mixed with 5 ml of scintillation fluid (Aquasol-2, Du Pont/New England Nuclear Research Products). The radioactivity level was determined using a liquid scintillation counter (LS5801, Beckman Instruments).

Immunoprecipitation and Western blotting. Cell lysates were immunoprecipitated and immunoblotted as described (24).

Electrophoretic mobility shift assay. To prepare nuclear extracts, cells were suspended in the hypotonic buffer (10 mM Hepes/KOH [pH 7.9], 10 mM KCl, 100 μM EDTA, 0.1% [v/v] Nonidet P-40, 1 mM dithiothreitol, 500 μM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM Na_3VO_4) and incubated for 10 min on ice. After a centrifuge at 3,000*g* for 1 min, the pelleted nuclei were resuspended in the extraction buffer (50 mM Hepes/KOH [pH 7.9], 420 mM KCl, 5 mM $MgCl_2$, 100 μM EDTA, 1 mM dithiothreitol, 500 μM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM Na_3VO_4, and 20% glycerol) and incubated for 30 min on ice. The lysates were centrifuged at 16,000*g* for 15 min, and resultant supernatants were used as nuclear extracts. Double-stranded oligonucleotides containing a consensus sequence for STAT1 or STAT3 (Santa Cruz Biotechnology) were labeled at 5′-ends with ^{32}P by T4 polynucleotide kinase, and purified using Sephadex G-50 (ProbeQuant G-50 Micro Columns, Amersham Pharmacia Biotech). For the DNA binding reactions, nuclear extracts (10 μg protein) were incubated with the labeled DNA probe (about 5×10^4 cpm) and poly(dI–dC) · poly(dI–dC) (2 μg, Amersham Pharmacia Biotech) in the binding buffer (12.5 mM Hepes/KOH [pH 7.9], 105 mM KCl, 1.25 mM $MgCl_2$, 250 μM EDTA, 25 μM dithiothreitol, 12.5 μM PMSF, 500 ng/ml aprotinin, 500 ng/ml leupeptin, 250 μM Na_3VO_4, and 5% glycerol) for 1 h on ice. For the competition experiments, a 100-fold molar excess of unlabeled oligomers was added prior to the addition of labeled probe. To identify the protein bound to DNA, nuclear extracts were preincubated with antibodies for 1 h prior to adding the labeled probe. Protein–DNA complexes were electrophoresed on 6% native polyacrylamide gel in 0.5× TBE buffer (1× TBE: 89 mM Tris, 89 mM boric acid, and 2 mM EDTA [pH 8.0]) at 4°C. Dried gels were analyzed for radioactivity using a bioimage analyzer BAS-2500 (Fuji Photo Film Co.).

Statistics. Results are expressed as means ± standard deviation of the number of observations. Statistical significance was assessed by Student's *t* test.

RESULTS

α_1 Agonist-Induced Tyrosine Phosphorylation of Jak2 and Tyk2

To examine whether the activation of Jak kinases is characteristic of angiotensin II signaling or α_1 receptor also induces their activation, we stimulated quiescent VSMCs with angiotensin II (100 nM) and the α_1 agonist phenylephrine (100 nM). As shown in Fig. 1a, not only angiotensin II but also phenylephrine induced phosphorylation of Jak2 and Tyk2. The maximal effects were obtained 5 min after stimulation, and the phosphorylation levels were significantly elevated until 1 h. The expression levels of Jak2 and Tyk2 were not influenced by the agonists. We could not detect an increase in the phosphorylation levels of Jak1 and Jak3 (not shown).

The agonist-specificity of the angiotensin II- and phenylephrine-induced Jak2 phosphorylation was tested using antagonists specific to their receptors (Fig. 1b). The AT_1 receptor antagonist candesartan but not the AT_2 receptor antagonist PD123319 inhibited angiotensin II-induced phosphorylation of Jak2, suggesting that AT_1 receptor but not AT_2 receptor is linked to Jak2 activation. α_1 receptor was bloked by prazosin, WB4101, an α_{1A} receptor specific antagonist, and CEC, an irreversible alkylating agent relatively specific to α_{1B} receptor. Phenylephrine-induced phosphorylation of Jak2 was inhibited by prazosin and CEC, but not by WB4101. Therefore, α_{1B} but not α_{1A} receptor seemed to be involved in this response.

The activation of Jak2 and Tyk2 was not limited to angiotensin II and phenylephrine, because endothelin-1 (100 nM) and thrombin (10 U/ml) also induced their phosphorylation (Fig. 1c).

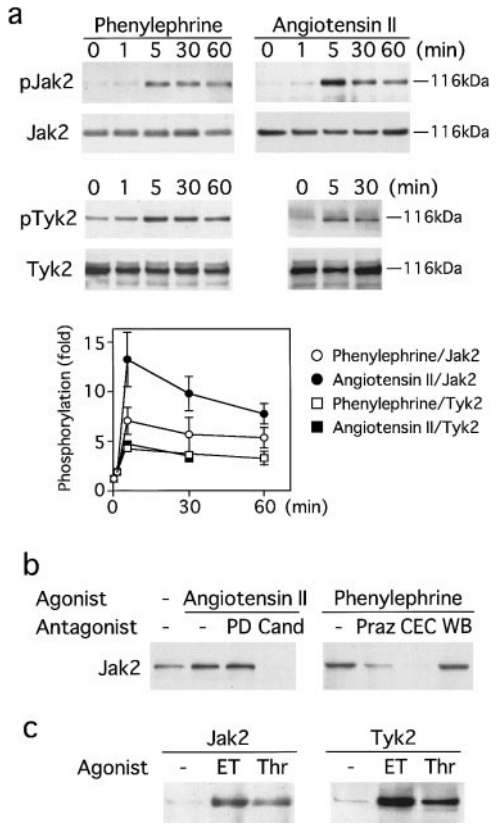

FIG. 1. Tyrosine phosphorylation of Jak2 and Tyk2 induced by G-protein-coupled receptor ligands. (a) Quiescent cells were stimulated with phenylephrine (100 nM) or angiotensin II (100 nM) for the periods indicated. Cell lysates were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody (PY20, Santa Cruz Biotechnology) or polyclonal antibodies to Jak2 and Tyk2 (Santa Cruz Biotechnology) and immunoblotted with the antibodies to Jak2 and Tyk2. pJak2, phosphorylated Jak2; pTyk2, phosphorylated Tyk2. The phosphorylation levels were quantified with an image analyzer and are shown as fold increase (n = 3). (b) Quiescent cells were stimulated with phenylephrine or angiotensin II for 5 min after the treatment with various receptor antagonists. Prazosin (Praz, 1 μM), WB4101 (WB, 1 μM), candesartan (Cand, 10 μM), and PD123319 (PD, 1 μM) were added 10 min prior to the stimulation with agonists. CEC (30 μM) was added 30 min prior to the agonist stimulation and washed out before cells were stimulated with phenylephrine. Tyrosine phosphorylation of Jak2 was analyzed as described in (a). (c) Quiescent cells were stimulated with endothelin-1 (ET, 100 nM) or thrombin (Thr, 10 U/ml) for 5 min. Tyrosine phosphorylations of Jak2 and Tyk2 were analyzed as described in (a).

α_1 Agonist-Induced Activation of STAT1

To determine whether α_1 stimulation leads to the activation of STAT proteins, we analyzed their tyrosine-phosphorylation after stimulating VSMCs with phenylephrine. As shown in Fig. 2a, phenylephrine induced phosphorylation of STAT1, with the maximal effect being obtained at 1 h. The expression level of STAT1 was not altered by the agonist. Since prazosin and AG490, a tyrphostin-derivative that selectively inhibits Jak2 (25), inhibited the phosphorylation of STAT1 induced by phenylephrine, this phosphorylation seemed to be mediated by α_1 receptor and Jak2 (Fig. 2b).

In the electrophoretic mobility shift assay, phenylephrine slowed the mobility of the consensus oligonucleotides for STAT1 as did interferon-γ, and this effect was inhibited by prazosin (Fig. 3). The phenylephrine-induced shift was also prevented by an anti-STAT1 antibody and by the preincubation of the nuclear extract with a 100-fold excess of unlabeled oligonucleotides. However, phenylephrine did not stimulate the DNA binding activity of STAT3 (not shown).

Phenylephrine-Induced Association of α_1 Receptor with Jak2 and STAT1

The phosphorylation of Jak2 induced by phenylephrine was unlikely to be mediated by *de novo* synthesized proteins, because it occurred quickly (within 5 min) after stimulation with the agonist (Fig. 1a), and moreover, phenylephrine was able to induce Jak2 phosphorylation even in the presence of CHX (Fig. 4a).

AT_1 receptor directly associates with Jaks and STATs (8). To examine whether α_1 receptor also phys-

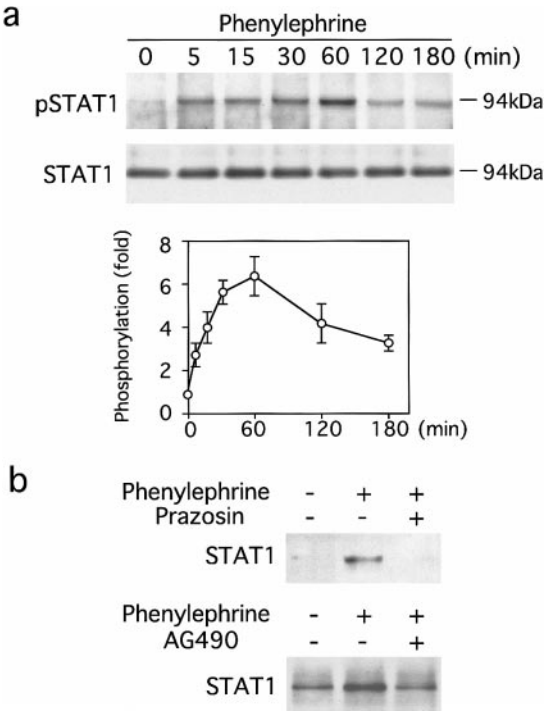

FIG. 2. Phenylephrine-induced phosphorylation of STAT1. (a) Quiescent cells were stimulated with phenylephrine (100 nM) for the periods indicated. Cell lysates were precipitated with PY20 or an anti-STAT1 polyclonal antibody (Santa Cruz Biotechnology) and blotted with the anti-STAT1 antibody. pSTAT1: phosphorylated STAT1. The results of quantitative analyses are also shown (n = 3). (b) Quiescent cells were stimulated with phenylephrine (100 nM) for 1 h. Prazosin (1 μM) and AG490 (3 μM) were added 10 min prior to the agonist stimulation. The phosphorylation of STAT1 was analyzed as described in (a).

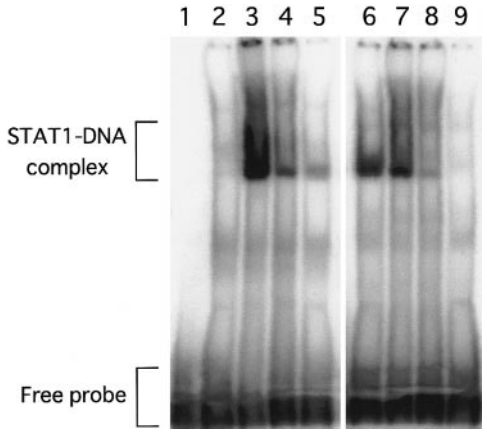

FIG. 3. Electrophoretic mobility shift assay for STAT1. Quiescent cells were stimulated with phenylephrine (100 nM) or interferon-γ (10 ng/ml) for 1 h. Prazosin (1 μM) was added 10 min before phenylephrine. Extracted nuclear proteins (10 μg) were incubated with ^{32}P-labeled double-stranded oligonucleotides containing a consensus sequence for STAT1. To identify the protein associating with DNA, some of the extracts were preincubated with an anti-STAT1 antibody (5 μg, Santa Cruz Biotechnology) or a nonimmunized IgG (5 μg). For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides was added prior to the addition of radiolabeled probes. Lane 1, no nuclear protein; 2, no agonist stimulation; 3, interferon-γ; 4–9, phenylephrine; 5, prazosin; 7, nonimmunized IgG; 8, anti-STAT1 antibody; 9, excess unlabeled oligonucleotides.

ically associates with these molecules, cell lysates were immunoprecipitated with anti-α_1 receptor antibodies and immunoblotted with anti-Jak2 and STAT antibodies. As demonstrated in Fig. 4b, the amounts of Jak2 and STAT1 coprecipitated with α_{1B} receptor increased in time-dependent manners, while the expression level of α_{1B} receptor was not significantly changed. However, STAT3 was not found in the α_{1B} receptor immunoprecipitates (not shown). Jak2 and STAT1 were not coprecipitated with α_{1A} or α_{1D} receptors (not shown).

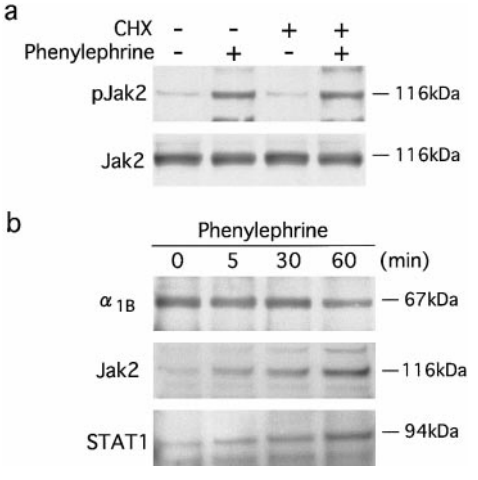

FIG. 4. Phenylephrine-induced association of α_{1B} receptor with Jak2, Tyk2, and STAT1. (a) Quiescent cells were stimulated with phenylephrine (100 nM) for 10 min in the absence or presence of CHX (3 μM), which was added 30 min prior to the agonist stimulation. Tyrosine phosphorylation of Jak2 was analyzed as described in the legend to Fig. 1. pJak2: phosphorylated Jak2. (b) Quiescent cells were stimulated with phenylephrine (100 nM) for the periods indicated. Cell lysates were precipitated with anti-α_{1B} receptor polyclonal antibody (Santa Cruz Biotechnology) and blotted with polyclonal antibodies to α_{1B} receptor, Jak2, and STAT1.

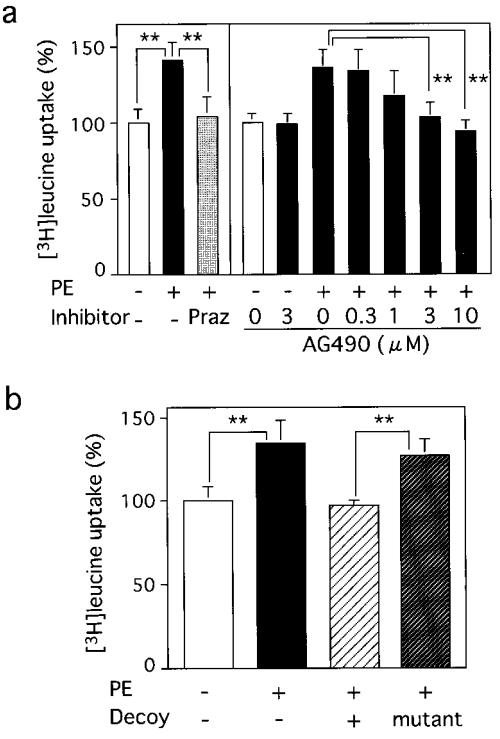

FIG. 5. Phenylephrine-stimulated protein synthesis. (a) Quiescent cells were stimulated with phenylephrine (PE, 100 nM) in the presence of [^{3}H]leucine (37 kBq/ml) for 24 h. Prazosin (Praz, 1 μM) and various concentrations of AG490 were added simultaneously with phenylephrine. (b) Cells transfected with the decoy oligonucleotide for STAT1 or its mutant were stimulated with phenylephrine and [^{3}H]leucine uptake was measured as in (a). Data are shown as percentages of the values obtained in unstimulated cells ($n = 3$). $^{**}P < .01$.

The Jak/STAT Pathway Mediates α_1 Agonist-Stimulated Protein Synthesis

Since α_1 adrenergic agonists have been shown to promote cellular hypertrophy in VSMCs and cardiac myocytes (17–19), we investigated whether the Jak/STAT pathway is involved in the hypertrophic response (Fig. 5). Phenylephrine promoted [^{3}H]leucine uptake by 1.3- to 1.4-fold. This stimulation was inhibited by prazosin and AG490 (Fig. 5a), suggesting that α_1 receptor and Jak2 mediate the hypertrophic response. To examine whether STAT1 is involved, we prepared cells transfected with a double-stranded oligonucleotide of the consensus sequence for STAT1 and compared their levels of [^{3}H]leucine uptake with those

in cells transfected with a mutant oligonucleotide. As shown in Fig. 5b, protein synthesis stimulated by phenylephrine was strongly suppressed in cells transfected with the decoy oligonucleotide.

In the meantime, phenylephrine and angiotensin II did not stimulate DNA synthesis (not shown).

DISCUSSION

The present study showed for the first time that not only AT_1 but also α_{1B} receptor is linked to the Jak/STAT pathway in VSMCs. The α_1 agonist-induced activation of this pathway may not be secondary to that by paracrine factors produced in response to the agonist, because Jak2 was phosphorylated immediately after stimulation with phenylephrine, secondly, CHX had no effect on the phosphorylation of Jak2, and thirdly, Jak2 was physically bound to α_{1B} receptor. There have been a few reports demonstrating the linkage between other types of G-protein-coupled receptors and this pathway. Thrombin stimulates Jak2 phosphorylation in platelets (26) and activates *sis*-inducing factor-A in VSMCs (27); Endothelin-1 activates *sis*-inducing factor in CHO-K1 cells expressing ET_A receptor (28); and α-melanocyte-stimulating hormone activates Jak2 and STAT1 in B-lymphocytes (29). Therefore, the Jak/STAT pathway is likely to be a common machinery shared among several G-protein-coupled receptors. It remains uncertain, however, whether this pathway is ubiquitously linked to seven-transmembrane receptors, because contradictory results also have been demonstrated in bombesin- and angiotensin II-stimulated cells (30).

In signal transduction of interleukin 6-related cytokines, gp130 is crucial to the activation of the Jak/STAT pathway (31). How do the G-protein-coupled receptors that do not associate with gp130 stimulate this pathway? Jak2 phosphorylation has been shown to be induced by Ca^{2+} ionophore and phorbol ester in platelets, suggesting that the activation of Jak2 is not an event independent of inositol phospholipid metabolism immediately induced after G-protein-coupled receptor stimulation (26). More recently, however, Jak2 has been reported to directly recognize the YIPP motif in the cytoplasmic tail of the AT_1 receptor (32). In the meantime, it has been hypothesized that STATs recognize tyrosine-phosphorylated AT_1 receptor with their SH2 domain and directly bind to the receptor protein (33). In our study, STAT1 was coprecipitated with α_{1B} receptor, in spite of the fact that α_{1B} receptor contains a STAT3 consensus motif (YSLQ) in the second cytoplasmic loop but no STAT1 consensus (YDXXH) (34). We examined whether STAT1 directly binds to α_{1B} receptor with its SH2 domain by the Westwestern method using glutathione S-transferase-fused STAT1 and its deletion mutants. However, we could not detect a phenylephrine-induced increase in the amount of the fusion proteins that bound to α_{1B} receptor transferred onto a membrane even if they contained SH2 domain (not shown). Further study is needed to elucidate the mechanism by which α_{1B} receptor transduces its signal to the Jak/STAT pathway.

In cardiac muscle hypertrophy induced by interleukin 6-related cytokines, STAT3 has been suggested to be involved in gp130-mediated signaling (23). STAT3 could be involved also in the angiotensin II-induced cardiac myocyte hypertrophy, since AT_1 receptor contains STAT3 consensus motifs (33). Our study suggested that VSMC hypertrophy induced by α_1 agonist also is mediated by the Jak/STAT pathway. In our cells, STAT1 is likely to be involved in the hypertrophic response, because phenylephrine induced STAT1 phosphorylation, it stimulated the DNA binding activity of STAT1, and protein synthesis was strongly inhibited by the decoy oligonucleotide for STAT1. However the involvement of STAT3 was unlikely, because phenylephrine did not elevate the DNA binding activity of STAT3 and did not stimulate the association of STAT3 with α_{1B} receptor. Our study also suggested that the receptor subtype linked to the Jak/STAT pathway is α_{1B} not α_{1A}, because prazosin and CEC but not WB4101 prevented Jak2 phosphorylation, and secondly α_{1B} but not α_{1A} or α_{1D} receptor was found to directly associate with Jak2. These results are consistent with previous studies demonstrating that α_{1B} receptor mediates hypertrophic response, and in contrast non-α_{1B} receptors attenuate this response (18, 19).

Medial thickening in hypertension is due in part to increased VSMC content, which occurs primarily by enlargement of preexisting VSMCs, with little or no change in the number of VSMCs (35). This suggests that VSMC hypertrophy is regulated differently from the proliferation. Our cells did not proliferate in response to phenylephrine or angiotensin II, although α_1 stimulation has been shown to promote DNA synthesis in VSMCs (36). There is discrepancy between studies on the role of the Jak/STAT pathway in cell proliferation. Marrero *et al.* suggested that STAT1 and STAT3 play an essential role in VSMC proliferation in response to angiotensin II (37). In contrast, Chin *et al.* showed that STAT1 mediates the antiproliferative signal of interferon-γ by inducing the expression of $p21^{Sdi1/Waf1/Cip1}$, a cyclin-dependent kinase inhibitor (38). Moreover, Pansky *et al.* demonstrated that antiproliferative cytokine interferon-α certainly activates the Jak/STAT pathway, whereas the proliferative response induced by bombesin and angiotensin II is not mediated by this pathway (30). Further study is needed to determine the role of the Jak/STAT pathway in cell proliferation.

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