Angiotensin II Stimulates Tyrosine Phosphorylation and Activation of Insulin Receptor Substrate 1 and Protein-tyrosine Phosphatase 1D in Vascular Smooth Muscle Cells

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Angiotensin II (Ang II) and insulin-like growth factor I (IGF I) stimulate intracellular signaling events through binding to their respective G-protein-coupled and growth factor receptors. In rat aortic vascular smooth muscle cells, IGF I (20 ng/mL) induced a sustained (>30 min) increase in the tyrosine phosphorylation of both Src-homology 2-domain-docking insulin receptor substrate 1 (IRS-1) and Src-homology 2-binding tyrosine phosphatase 1D (PTP-1D). In addition, IGF I stimulated PTP-1D phosphatase activity. Ang II (10^{-7} M) also increased the tyrosine phosphorylation of IRS-1 (4-fold), PTP-1D (5-fold), and PTP-1D activity (3–4-fold), but with a more transient time course. Ang II also induced PTP-1D-IRS-1 complex formation. These Ang II-induced events were not affected by preincubation with an anti-IGF I antibody, suggesting that Ang II's actions were not mediated via the autocrine secretion of IGF I. Anti-PTP-1D antibody electroploration attenuated Ang II-induced PTP-1D-IRS-1 complex formation and PTP-1D tyrosine phosphorylation and activation. Our findings show that the tyrosine phosphorylation of IRS-1 and PTP-1D represents a convergent intracellular signaling cascade stimulated by both growth factor (i.e. IGF I) and G-protein-coupled (i.e. AT1) receptors.

The octapeptide angiotensin II (Ang II) is the major effector molecule of the renin-angiotensin system (10, 11). One of its primary roles is to regulate vascular tone through contractile actions on vascular smooth muscle cells (VSMC). However, Ang II also has mitogenic effects on VSMC including hypertrophic growth (1, 2), stimulation of platelet-derived growth factor expression (3), induction of proto-oncogenes c-fos and c-jun (4), and stimulation of protein tyrosine phosphorylation (5). Ang II stimulates phospholipase C-γ1 and the hydrolysis of phosphati-dylinositol 4,5-bisphosphate, yielding the second messen-gers inositol 1,4,5-trisphosphate and diacylglycerol (7). Inositol 1,4,5-trisphosphate mobilizes sequestered stores of calcium (8), whereas diacylglycerol stimulates protein kinase C (9).

The VSMC Ang II receptor has been cloned and is now designated as AT1 (6). The AT1 receptor is a seven-transmembrane-spanning receptor coupled to heterotrimeric G-proteins and is responsible for virtually all the physiological actions of Ang II (10, 11). Tyrosine phosphorylation and dephosphorylation of cellular proteins is now well recognized as a critical event in intracellular signal transduction (19, 20). Although G-protein-coupled receptors do not possess intrinsic tyrosine kinase activity, a recently developed concept is that they acti-vate intracellular second messenger proteins through tyrosine phosphorylation by cytosolic protein tyrosine kinases (12). Indeed, recent studies using various cell types, including VSMC, have demonstrated that Ang II stimulates the tyrosine phosphorylation of pp125Fak, pp120, JAK2, STAT1, STAT2, SHC, pp60-src, paxillin, phospholipase C-γ1, TYK2, and p44MAPK.

Classic growth factors mediate their effects via receptors that possess intrinsic tyrosine kinase activity (19, 20). Insulin-like growth factor I (IGF I), an important autocrine/paracrine factor for VSMC, activates a specific heterotetrameric G-proteins and is responsible for virtually all the physiological actions of Ang II (10, 11). Tyrosine phosphorylation and dephosphorylation of cellular proteins is now well recognized as a critical event in intracellular signal transduction (19, 20). Although G-protein-coupled receptors do not possess intrinsic tyrosine kinase activity, a recently developed concept is that they activate intracellular second messenger proteins through tyrosine phosphorylation by cytosolic protein tyrosine kinases (12). Indeed, recent studies using various cell types, including VSMC, have demonstrated that Ang II stimulates the tyrosine phosphorylation of pp125Fak, pp120, JAK2, STAT1, STAT2, SHC, pp60-src, paxillin, phospholipase C-γ1, TYK2, and p44MAPK.

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* The abbreviations used are: Ang II, angiotensin II; VSMC, vascular smooth muscle cells; IGF I, insulin-like growth factor I; IRS-1, insulin receptor substrate 1 (IRS-1) and Src-homology 2-binding tyrosine phosphatase 1D; PTP-1D, protein-tyrosine phosphatase; PTPase, protein-tyrosine phosphatase; PTP-1D, PTPase 1D; DMEM, Dulbecco's modified Eagle’s medium; GST, glutathione S-transferase; [U], the amount of p-nitrophenol in micromoles released per minute.

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EXPERIMENTAL PROCEDURES

Materials—TWEEN 20, acrylamide, SDS, N,N'-methylene-bisacrylamide, N,N',N',N'-tetramethylethylenediamine, and nitrocellulose membranes were purchased from Bio-Rad. Insulin-like growth factor I, molecular weight standards, immunoprecipitin, protein A- and G-agarose, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, trypsin, and all medium additives were obtained from Life Technologies, Inc. Monoclonal antibodies to phosphotyrosine were obtained from Transduction Laboratories (P2Y0) and Upstate Biotechnology, Inc. (4G10). Anti-IRS-1 and anti-PTP-1D antibodies were obtained from either Santa Cruz Biotechnology, Inc. or Transduction Laboratories. Anti-IGF-I antibody was kindly provided by D. Pelafontaine. Glutathione-S-transferase (GST)-PTP-1D fusion protein was obtained from Santa Cruz Biotechnology, Inc. The enhanced chemiluminescence kit was obtained from Amersham Corp. Antibodies against mouse IgG, and all other chemicals were purchased from Sigma.

Cell Culture—VSMC from 200–300-g male Harlan Sprague Dawley rat aortas were isolated and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 10 mg/ml streptomycin, and 100 units/ml of penicillin and gentamicin. Growth-arrested VSMC were electroporated in tissue culture dishes using a Petri dish electrode (100 mm in diameter, a 2-mm gap) manufactured by BTX Inc. (14). Electroporation was performed in Ca2+-free and Mg2+-free Hanks’ balanced salt solution (5 mM KCl, 3.0 mM KH2PO4, 139 mM NaCl, 60 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM N-acetyl-L-cysteine, 10 mM NaHCO3, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM µg/ml apamin, 100 μM µg/ml leupeptin, pH 7.4). Electroporation was performed by guest on July 26, 2018http://www.jbc.org/Downloaded from

RESULTS

Ang II and IGF I Induce the Tyrosine Phosphorylation of IRS-1—VSMC were exposed to the G-protein-coupled receptor ligand Ang II (10−7 m) for 0, 1, 5, 10, or 30 min and then IRS-1 tyrosine phosphorylation was measured. Cell lysates were immunoprecipitated with a monoclonal anti-phosphotyrosine antibody. The precipitated proteins were then separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with a polyclonal anti-IRS-1 antibody. Similar results were obtained when the order of antibody addition was reversed, i.e. when the cell lysate was immunoprecipitated with anti-IRS-1 antibody and then immunoblotted with an anti-phosphotyrosine antibody (n = 4; data not shown). Fig. 1A shows that the levels of IRS-1 tyrosine phosphorylation were significantly increased within 1 min of Ang II exposure, peaked at 5 min, and returned to near basal values by 10 min. In a separate set of experiments, VSMC were again exposed to Ang II, but this time in the presence of the AT1 receptor antagonist losartan (10−5 m); (34). No increase in Ang II-induced tyrosine phosphorylation of IRS-1 was detected under these conditions, consistent with previous reports that expression of the AT1 receptor isoform predominates in VSMC (n = 3; data not shown) (35).

Similar experiments were performed with VSMC exposed to the classic growth factor IGF I (20 ng/ml) for timed periods. IGF-I is a well established inducer of IRS-1 tyrosine phosphorylation (22, 23). Peak tyrosine-phosphorylation responses again occurred within 1 min of IGF I exposure (Fig. 1B). However, in contrast to the transient effect of Ang II, IRS-1 tyrosine phosphorylation was still significantly increased even after 30 min of IGF I exposure. Therefore, in VSMC the Ang II-induced tyrosine phosphorylation of IRS-1 is induced by both the G-protein-coupled AT1 receptor and the IGF I growth factor receptor, albeit with much different time courses.

Ang II and IGF I Induce the Tyrosine Phosphorylation and PTPase Activity of PTP-1D—Ang II-induced tyrosine phospho-
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Tyrosine phosphorylation of IRS-1 by Ang II and IGF I.

VSMC cells were stimulated with either Ang II (10^{-7} M) (inset A and ○) or IGF I (20 ng/ml) (inset B and ○) for 0, 1, 5, 10, or 30 min. Cell lysates were immunoprecipitated with an anti-IRS-1 antibody and probed with anti-phosphotyrosine antibody. The insets show representative bands corresponding to the molecular mass of IRS-1 (160–185 kDa). Bands were quantitated by densitometry using a La Cie scanner interfaced with a personal computer. Each band was scanned in two dimensions and the density corrected for the background present in the lane. The graph represents the corrected density for each time point, and data are expressed as arbitrary units plotted against time of Ang II or IGF I exposure (mean ± S.E.; n = 4).

Tyrosine phosphorylation of PTP-1D by Ang II and IGF I.

VSMC cells were stimulated with either Ang II (10^{-7} M) (inset A and ○) or IGF I (20 ng/ml) (inset B and ○) for 0, 1, 5, 10, or 30 min. Cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody and probed with anti-PTP-1D antibody. The insets show representative bands corresponding to the molecular mass of PTP-1D (72 kDa). The graph represents the corrected density for each time point, and data are expressed as arbitrary units plotted against time of Ang II or IGF I exposure (mean ± S.E.; n = 4).

PTP-1D enzymatic activity in response to Ang II and IGF I.

PTP-1D activity was also measured in response to IGF I, and again we observed a temporal correlation between the IGF I-induced increase in PTP-1D activity and the tyrosine phosphorylation of the phosphatase (Fig. 3). We have previously shown that many Ang II-induced tyrosine phosphorylation events are mediated through a cytosolic Src tyrosine kinase and are inhibited by genistein, a tyrosine kinase inhibitor (7). Therefore, we examined whether PTP-1D does indeed form a complex with IRS-1 in VSMC and whether this was an Ang II-dependent event. Immunoprecipitates, obtained from Ang II-stimulated VSMC with anti-IRS-1 antibody, were immunoblotted with an anti-PTP-1D antibody after SDS-polyacrylamide gel electrophoresis separation. A band with an apparent molecular mass similar to that of PTP-1D (72 kDa) was detected at 10 min and returned to near basal levels by 30 min. IRS-1 tyrosine phosphorylation at 10 min (Fig. 3; data not shown). IGF I also induced a 5-fold stimulation of PTP-1D tyrosine phosphorylation at 10 min (Fig. 2B). However, as with IRS-1 tyrosine phosphorylation, the temporal response to IGF I was more sustained than that observed with Ang II. The levels of tyrosine phosphorylation of PTP-1D were still significantly elevated after 30 min of IGF I exposure. To correlate the tyrosine phosphorylation of PTP-1D with the actual PTPase activity of PTP-1D, we measured the time course for Ang II-induced formation of p-nitrophenol from p-nitrophenylphosphate (see “Experimental Procedures” for details) (Fig. 3). p-Nitrophenol production was maximal between 5 and 10 min of Ang II exposure, a time course paralleling that of Ang II-induced tyrosine phosphorylation of PTP-1D (Fig. 2A). As expected, p-nitrophenol formation in response to Ang II was mediated through the AT1 receptor, since the response was blocked by losartan (10^{-5} M) (n = 3; Fig. 3, black triangle). The peak response was observed at 10 min and returned to near basal levels by 30 min. Ang II also increased the levels of tyrosine-phosphorylated proteins detected by first immunoprecipitating with anti-PTP-1D antibody and then probing with anti-phosphotyrosine antibody (n = 3; data not shown). IGF I also induced a 5-fold stimulation of PTP-1D tyrosine phosphorylation at 10 min (Fig. 2B). The temporal response was observed at 10 min and returned to near basal levels by 30 min. Ang II also increased the levels of tyrosine-phosphorylated proteins detected by first immunoprecipitating with anti-PTP-1D antibody and then probing with anti-phosphotyrosine antibody (n = 3; data not shown). IGF I also induced a 5-fold stimulation of PTP-1D tyrosine phosphorylation at 10 min (Fig. 2B). However, as with IRS-1 tyrosine phosphorylation, the temporal response to IGF I was more sustained than that observed with Ang II. 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was unaffected by electroporation with rabbit IgG but was treated at time 0 and increased in intensity after Ang II exposure (Fig. 4). The time course for complex formation between PTP-1D and IRS-1 correlated with the Ang II-induced tyrosine phosphorylation of IRS-1 (Fig. 1A) and was consistent with the binding of PTP-1D to the tyrosine-phosphorylated form of IRS-1.

We also attempted to detect PTP-1D-IRS-1 complex formation by immunoprecipitating PTP-1D with anti-PTP-1D antibody and then probing with anti-IRS-1 antibody. However, no band corresponding to the molecular mass of IRS-1 (160–185 kDa) was detected using the latter protocol. The anti-PTP-1D antibodies used in this study were raised against the amino-terminal regions of PTP-1D, which contain the PTPase’s SH2 domains (Transduction Laboratories and Santa Cruz Biotechnology, Inc., certificates of analysis). Since the tyrosine-phosphorylated form IRS-1 also binds to SH2 domains, conceivably the anti-PTP-1D antibody may have prevented the binding of IRS-1 to antibody-bound PTP-1D by stearic interference. Therefore, we immunoprecipitated PTP-1D with a commercially available GST-PTP-1D fusion protein and probed with an anti-IRS-1 antibody. We reasoned that the GST-PTP-1D fusion protein would not interfere with SH2-domain binding between PTP-1D and IRS-1. Indeed, under these conditions we again observed Ang II-induced PTP-1D-IRS-1 complex formation (Fig. 5).

Effects of Electroporated Anti-PTP-1D Antibody on Ang II-stimulated PTP-1D Tyrosine Phosphorylation, PTPase Activity, and IRS-1 Complex Formation—We have previously shown in VSMC that electrophoresis of specific antibodies against cellular messenger proteins is an effective means of interrupting Ang II-induced signal transduction cascades (14). Our above observation that anti-PTP-1D antibody stereically interfered with PTP-1D and IRS-1 binding prompted us to investigate the effects of blocking PTP-1D-IRS-1 complex formation on the Ang II-induced tyrosine phosphorylation and activation of PTP-1D. VSMC were electroporated with anti-PTP-1D antibody and then treated with 10−7 M Ang II for timed periods. Ang II-induced complex formation between PTP-1D and IRS-1 was indeed significantly blocked in VSMC electroporated with anti-PTP-1D antibody (Fig. 4). On the other hand, electroporation of rabbit IgG (mock electroporation) had no effect on complex formation (Fig. 4).

We then measured the tyrosine phosphorylation of PTP-1D in VSMC electroporated with either anti-PTP-1D antibody or rabbit IgG and then exposed to 10−7 M Ang II (Fig. 6). VSMC lysates were again immunoprecipitated with a monoclonal anti-phosphotyrosine antibody and probed with anti-PTP-1D antibodies. Ang II-induced tyrosine phosphorylation of PTP-1D was unaffected by electroporation with rabbit IgG but was abolished in VSMC electroporated with anti-PTP-1D antibody (Fig. 6). Predictably, we also confirmed that electrophoresis of anti-PTP-1D antibody into VSMC significantly inhibited the Ang II-induced PTPase activity of PTP-1D (Fig. 7). Conversely, the electroporation of the cells with rabbit IgG had no effect on PTP-1D activity. These results suggested that tyrosine phosphorylation and activation of PTP-1D by Ang II was dependent on complex formation between PTP-1D and IRS-1.

Effect of Anti-IGF I Antibody on the Ang II-induced Tyrosine Phosphorylation of IRS-1 and Activation of PTP-1D—We examined the possibility that our observed Ang II-induced responses were mediated secondary to autocrine secretion of IGF I, which has been shown by Delafontaine and Lou (44) to occur in VSMC. Ang II-induced tyrosine phosphorylation of IRS-1 and PTP-1D activity were again measured but this time in VSMC preincubated with normal rabbit serum or anti-IGF I antibody (1:500 dilution) for 30 min. In the presence of anti-IGF I antibody, PTP-1D activation (Fig. 8) and the tyrosine phosphorylation of PTP-1D (data not shown) and IRS-1 (Fig. 9) in response to Ang II were unaffected. In another set of experiments, VSMC were first pretreated with Ang II for 5 or 10 min and then the extracellular medium was extracted. We then incubated VSMC in this Ang II-conditioned medium plus 10−5 M losartan (n = 3). Under these conditions, there was no increase in IRS-1 and PTP-1D tyrosine phosphorylation or PTPase activity of PTP-1D (data not shown). These results suggested that the responses observed in this study are due to a direct effect of Ang II rather than a secondary effect mediated by the autocrine release of IGF I or some other unidentified cytokine into the medium. As previously shown by Delafontaine and Lou (44), we confirmed that preincubation of VSMC with anti-IGF I antibody still blocked Ang II-induced DNA.
synthesis measured by $[^3]$H]thymidine incorporation ($n = 3$; data not shown).

**DISCUSSION**

In this study, we demonstrate that both the vasoactive peptide, Ang II, and the classic growth factor, IGF I, induce the tyrosine phosphorylation of the SH2-docking protein, IRS-1, and the SH2-containing tyrosine phosphatase, PTP-1D. In addition, the two ligands also stimulate the PTPhase activity of PTP-1D. However, we find that the responses of IRS-1 and PTP-1D tyrosine phosphorylation and PTP-1D activation are temporally different when the VSMC are exposed to Ang II rather than IGF I. Although Ang II-induced responses are transient, returning to baseline in 10 to 30 min, IGF I-induced responses are sustained, still significantly elevated even after 30 min of ligand exposure.

We also demonstrate that Ang II induces the formation of a complex between PTP-1D and IRS-1 by either co-immunoprecipitation with an anti-IRS-1 antibody or co-precipitation with a GST-PTP-1D fusion protein. However, there are some discrepancies in the kinetics of complex formation between these two methods. Anti-IRS-1 co-immunoprecipitation shows a more prolonged association that is maximal at 10 min, whereas GST-PTP-1D fusion protein co-precipitation shows a more transient association that is maximal at 5 min. These differences might be due merely to nonspecific interactions with the anti-IRS-1 antibody or the protein A/G plus agarose during the co-immunoprecipitation procedure. Alternatively, the co-precipitation procedure with the fusion protein may not accurately
reflect all of the complex interactions that occur between IRS-1 and PTP-1D. Other regions of the PTP-1D molecule not present in the fusion protein may also play important roles in the protein-protein interactions between IRS-1 and PTP-1D. In any event, both procedures do document that PTP-1D-IRS-1 complex formation is blocked when the PTP-1D SH2-binding site for IRS-1 is blocked by electroporated anti-PTP-1D antibody. In addition, interference of PTP-1D-IRS-1 complex formation by anti-PTP-1D antibody also inhibits the Ang II-induced tyrosine phosphorylation and activation of PTP-1D. Therefore, our data suggest that stimulation of PTP-1D phosphatase activity by Ang II is dependent on PTP-1D association with IRS-1. In turn, these events are dependent on tyrosine phosphorylation, since genistein blocks both Ang II-induced tyrosine phosphorylation and activation of PTP-1D.

Previous work by Delafontaine and Lou (44) has shown that Ang II can increase VSMC IGF I mRNA expression and IGF I secretion with peak responses after 6 h of Ang II exposure. This time course is much more chronic than the Ang II- and IGF I-induced responses observed in the present study, which peaked after 5–10 min of ligand exposure. Nevertheless, we examined the possibility that our observed Ang II-induced responses were mediated secondary to autocrine secretion of IGF I. We preincubated VSMC for 30 min with a polyclonal anti-IGF I antibody (1:500 dilution) that was raised in rabbits against human recombinant IGF I and that has been previously shown by Western immunoblotting and enzyme-linked immunosorbent assay to have no cross-reactivity with either insulin or IGF II (34). Delafontaine and Lou (44) have also shown that this anti-IGF I antibody neutralizes up to 100 ng/ml IGF I at a 1:500 dilution. In the presence of anti-IGF I antibody, Ang II-induced tyrosine phosphorylation of PTP-1D and IRS-1 and activation of PTP-1D was unaffected. However, preincubation of VSMC with anti-IGF I antibody still blocked Ang II-induced DNA synthesis, as previously shown by Delafontaine and Lou (44). We also incubated cells in Ang II-conditioned medium from VSMC plus 10−5 M losartan and observed no stimulation of tyrosine phosphorylation of IRS-1 and PTP-1D or PTPase activity of PTP-1D. Therefore, in VSMC the effect of Ang II on tyrosine phosphorylation of IRS-1 and PTP-1D and PTP-1D activity appears to be direct and not merely secondary to the autocrine release of IGF I or some other unidentified cytokine into the medium.

In VSMC, Ang II has recently been shown to stimulate the transient phosphorylation of tyrosine residues on various proteins (7, 13–18). The transient nature of these Ang II-induced tyrosine phosphorylation events highlights the potential importance of PTPases in the regulation of Ang II-mediated cellular signaling events (36). For example, in hematopoietic cells tyrosine dephosphorylation is involved in the activation of the cytosolic tyrosine kinase pp60c-src (37). We have previously shown that many Ang II-induced tyrosine phosphorylation events are mediated by pp60c-src in VSMC (7, 13–15).

PTPases are evolutionarily conserved, are widely distributed in nature, and are present in both normal and neoplastic cells (39). These enzymes are involved in terminating the levels of protein tyrosine phosphorylation in cells. It has been proposed that altered tyrosine dephosphorylation of proteins leads to neoplastic transformation (38). Characterization of these enzymes shows that they can be divided into two types: low molecular mass (<80 kDa) cytosolic PTPases and high molecular mass (>80 kDa) membrane-associated (transmembrane) PTPases (39). The PTPases of the cytosolic type (e.g. PTP-1D) have a single phosphatase domain, whereas those of the transmembrane type have two phosphatase domains. PTPases participate in the regulatory mechanism of cell proliferation and differentiation, and some PTPases are also involved in signal transduction. We have recently demonstrated that Ang II stimulates the mRNA expression and protein synthesis of the immediate early response gene 3CH134, which encodes a PTPase (37). We have previously demonstrated that the Ang II-mediated induction of 3CH134 is dependent on intracellular Ca2+ levels and is partially dependent on protein kinase C activity (40).

No other specific PTPase, other than 3CH134, has previously been shown to be activated by Ang II in VSMC. In the present study, we demonstrate that the levels of tyrosine phosphorylation and activation of PTP-1D phosphatase activity are dramatically increased by Ang II and that these Ang II-induced effects are blocked by the AT1 receptor antagonist losartan in VSMC. Others have recently shown that PTP-1D is constitutively tyrosine phosphorylated in v-src-transformed cells (41). Indeed, we have recently demonstrated that Ang II stimulates the kinase activity of pp60c-src via the AT1 receptor (15) and that Ang II-induced pp60c-src stimulates protein tyrosine phosphorylation (e.g. phospholipase C-γ1) in VSMC (14). Thus, Ang II may promote the tyrosine phosphorylation of PTP-1D in VSMC by activating the tyrosine kinase pp60c-src. In a previous study, we have also found that preincubation of VSMC with 0.1 mM sodium orthovanadate (Na3VO4), a well characterized PTPase inhibitor (42), significantly augments Ang II-stimulated tyrosine phosphorylation events (e.g. activation of phospholipase C-γ1) (43). Therefore, these results reinforce the assertion that activation of PTPases is crucial for the regulation of signal transduction cascade associated with Ang II binding to the AT1 receptor in VSMC.

In summary, we have shown that receptors with different biochemical and structural characteristics can elicit similar early cellular signaling responses in VSMC. Specifically, the seven-transmembrane-spanning G-protein-coupled AT1 recep-
tor and the one-transmembrane-spanning tetrameric insulin-like growth factor I receptor both activate similar intracellular signaling proteins and enzymes, albeit with different time courses. Furthermore, we present the first example of a G-protein-coupled receptor (i.e. AT1 receptor) stimulating the rapid activation of the cytosolic tyrosine phosphatase PTP-1D.

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