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Involvement of c-Src Tyrosine Kinase in SHP-1 Phosphatase Activation by Ang II AT_2 Receptors in Rat Fetal Tissues

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ABSTRACT

Angiotensin II (Ang II) AT_2 receptors are abundantly expressed in rat fetal tissues where they probably contribute to development. In the present study we examine the effects of Ang II type 2 receptor stimulation on SHP-1 activation. Ang II (10^{-7} M) elicits a rapid and transient tyrosine phosphorylation of SHP-1, maximal at 1 min, in a dose-dependent form, blocked by the AT_2 antagonist, PD123319. SHP-1 phosphorylation is followed in time by tyrosine dephosphorylation of different proteins, suggesting a sequence of events. Ang II induces association of SHP-1 to AT_2 receptors as shown by co-immunoprecipitation, Western blot and binding assays. SHP-1 activity was determined in immunocomplexes obtained with either anti-AT_2 or anti-SHP-1 antibodies, after Ang II stimulation (1 min), in correlation with the maximal level of SHP-1 phosphorylation. Interestingly, following receptor stimulation (1 min) c-Src was associated to AT_2 or SHP-1 immunocomplexes. Preincubation with the c-Src inhibitor PP2 inhibited SHP-1 activation and c-Src association, thus confirming the participation of c-Src in this pathway. We demonstrated here for the first time the involvement of c-Src in SHP-1 activation via AT_2 receptors present in an ex vivo model expressing both receptor subtypes. In this model, AT_2 receptors are not constitutively associated to SHP-1 and SHP-1 is not constitutively activated. Thus, we clearly establish that SHP-1 activation, mediated by the AT_2 subtype, involves c-Src and precedes protein tyrosine dephosphorylation, in rat fetal membranes. J. Cell. Biochem. 105: 703–711, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: ANG II; AT_2 RECEPTORS; SHP-1 ACTIVATION; DEVELOPMENT

In addition to being a major regulator of blood pressure, the octapeptide Angiotensin II (Ang II) is a modulator of cellular growth. Ang II exerts a positive or negative effect on cell growth depending on which subtype of receptor is activated. The possible role of Ang II as a growth promoting or modulating factor has recently become a field of intensive research [Deshayes and Nahmias, 2005; Landon and Inagami, 2005; Godeny and Sayeski, 2006; Hunyady and Catt, 2006].

Ang II acts on its target tissues through binding to membrane receptors. Two main subtypes of Ang II receptors have been described, AT_1 receptors specifically blocked by Losartan (Los) and AT_2 receptors selectively displaced by CGP42112 or PD123319 [De Gasparo et al., 2000; Gallinat et al., 2000]. Both receptor subtypes belong to the superfamily of G-protein coupled receptors [Murphy et al., 1991; Sasaki et al., 1991; Mukoyama et al., 1993; Kambayashi et al., 1993] but they induce opposite biological effects, including vasoconstriction and cell growth [Horiuchi et al., 1999; Inagami et al., 1999], through different intracellular signaling pathways.

The multiple transduction pathways of AT_1 receptors have been recently reviewed [Hunyady and Catt, 2006; Higuchi et al., 2007]. The AT_1 subtype interacts with multiple heterotrimeric G-proteins, including $G_{q/11}$, G_i, G_{12} and G_{13} and produces different physiological effects [Higuchi et al., 2007]. AT_1 receptors have the ability to activate various intracellular kinases, such as tyrosine kinases and serine/threonine kinases, the MAPK family, including p38MAPK, Akt/PKB and various isoforms of PKC [Yin et al., 2003; Godeny and Sayeski, 2006; Hunyady and Catt, 2006; Kang et al., 2006; Higuchi et al., 2007]. The AT_1 subtype mediates transactivation of the epidermal growth factor (EGF) receptor [Eguchi et al., 1998],

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promotes tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and its association with the tyrosine phosphatase SHP-2 [Ali et al., 1997]. In rat liver, AT_1 receptors inhibit insulin-induced tyrosine phosphorylation of IRS-4, suggesting transactivation of insulin receptors [Villarreal et al., 2006].

The AT_2 receptor subtype is much less well characterized, and its physiological role is not completely understood [De Gasparo et al., 2000; Gallinat et al., 2000; Landon and Inagami, 2005; Li et al., 2007]. AT_2 receptors are abundantly expressed in fetal tissues and immature brain, but they are present only at low levels in certain adult tissues [De Gasparo et al., 2000; Gallinat et al., 2000]. The specific localization of AT_2 receptors in embryonic and neonatal tissues suggests a possible role of these receptors in growth and organogenesis [Arce et al., 2001]. Although their physiological functions are not well-established, it is now accepted that AT_2 receptors mediate anti-growth and apoptotic actions of Ang II [Deshayes and Nahmias, 2005].

The AT_2 receptor activates unconventional signaling pathways, which in most cases do not involve coupling to classical regulatory G-proteins [Nouet et al., 2004; Landon and Inagami, 2005]. AT_2 signaling pathways in relation to cell growth and differentiation are still under revision, although increasing evidence supports a role mediated by coupling to protein tyrosine phosphatases (PTPs) [Matsubara et al., 2001; Cui et al., 2001; Feng et al., 2002, 2005; Gendron et al., 2003; Li et al., 2007]. Recently, we have shown that Ang II stimulates a rapid tyrosine (Tyr) dephosphorylation of several proteins in rat whole fetal (E20) tissue preparations [Ciuffo et al., 1998] and in rat fetal kidney [Alvarez et al., 2003]. AT_2 receptor stimulation also induces neurite outgrowth and regulates neurofilaments in neural cell lines, NG108-15 cells, PC12W cells and fetal brain [Gendron et al., 2003; Li et al., 2007]. Interestingly, over expression of Ang II AT_2 receptors in CHO cells leads to induction of apoptosis independent of receptor conformation and ligand stimulation [Miura and Karnik, 2000; Miura et al., 2005]. On the other hand, negative cross-talk between Ang II AT_2 receptor subtype and insulin receptors has been recently reported [Elbaz et al., 2000].

Several interacting proteins of AT_2 receptors, such as AT_2 receptor interacting protein (ATIP) [Nouet et al., 2004] and SHP-1 have been described as partners of AT_2 receptor signaling [Landon and Inagami, 2005]. SHP-1 (also named as SHPTP-1, SHP or HCP) is a non-transmembrane PTPase that contains two Src homology 2 (SH2) domains involved in its association with multiple signaling molecules [Bousquet et al., 1998; Feng et al., 2002, 2005]. SHP-1 can be either phosphorylated or dephosphorylated in response to activation of G-protein coupled receptors, such as the sst2 Somatostatin receptor and the AT_2 Ang II receptor [López et al., 1997; Bousquet et al., 1998; Cui et al., 2001; Li et al., 2007].

SHP-1 is predominantly expressed in hematopoietic cells, generally acting as a negative regulator [Neel et al., 2003]. SHP-1 inhibits cell proliferation and mitogenic signals by dephosphorylation of critical molecules [Kozlowski et al., 1998]. Evidence suggests a negative role of SHP-1 on proliferative pathways [López et al., 1997; Bousquet et al., 1998; Kozlowski et al., 1998; Cui et al., 2001; Matsubara et al., 2001; Neel et al., 2003]. There is evidence that c-Src and SHP-1 are closely related and both participate in the regulation of phosphorylation sequences [Somani et al., 1997; Roskoski, 2004]. Based on enzymatic data and modeling studies, Frank et al. [2004] proposed that c-Src and SHP-1 might have complementary substrate selectivity.

In the present study we show that Ang II-induced protein tyrosine dephosphorylation in rat fetal membranes (E20) is mediated by the AT_2 receptor subtype. We provide evidence that Ang II stimulation leads to physical association of AT_2 receptors with SHP-1, as well as phosphorylation and functional activation of the SHP-1 tyrosine phosphatase. Co-immunoprecipitation assays demonstrate a concomitant association of AT_2 receptors and SHP-1 to c-Src kinase, and the use of specific Src inhibitor PP2 further suggests a role for this cytosolic tyrosine kinase in the AT_2/SHP-1 signaling pathway in rat fetal membranes.

MATERIALS AND METHODS

MATERIALS

Drugs were obtained from commercial suppliers: Bovine serum albumine, BSA fraction V, Triton X-100 and Sodium orthovanadate were from Sigma Chemical Co. (St. Louis, MO). Ang II, PP2 and PP3 were from Calbiochem (La Jolla, CA); Losartan was a gift from Dr. R. Smith (Dupont, Wilmington, DE) and PD123319 was derived from RBI (Natick, MA). [^{125}I]Sar^{1}Ile^{8}Ang II was from Dupont-NEN (Boston, MA). Polyvinylidene difluoride membranes (PVDF) were Immobilon P (Millipore Corp., Bedford, MA). CHAPS was purchased from J.T.Baker (Phillipsburg, NY). All other reagents were from the highest available quality.

Rabbit polyclonal anti-SHP-1 antibody was produced as described [Bedecs et al., 1997]. Rabbit anti-c-Src polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was a gift from Dr. JM Saavedra. PY99 mouse monoclonal anti-phosphotyrosine antibody, goat polyclonal anti-AT_2, mouse anti-SHP-1 antibody and secondary antibodies were purchased from (Santa Cruz Biotechnology)

ANIMALS

Adult female Wistar rats were kept in a dark-light cycle (12:12 h) and provided with standard rat food and water ad libitum. Day 0 of pregnancy was defined by sperm presence in vaginal smears. Pregnant rats were sacrificed by decapitation at day 20 and the fetuses immediately removed. Fetuses (E20) were sacrificed by decapitation, the viscera were removed and tissues for membrane preparation were collected on hypotonic buffer (50 mM Tris–HCl, 5 mM EDTA, pH 7.4) at 4°C and processed as below.

MEMBRANE PREPARATION

For membrane preparation tissues were homogenized in 40 volumes of hypotonic buffer using an Ultraturrax T25 homogenizer (Janke & Kunkel KG, IKA-Wak D7813 Staufer) 3 times, 10 s each, as described [Alvarez et al., 2003]. The homogenate was centrifuged at 1,000*g* for 10 min to separate nuclear and cellular debris. The supernatant was centrifuged at 15,000*g* for 60 min. The pellet was washed twice on hypotonic buffer and resuspended in isotonic buffer (50 mM Tris–HCl, 5 mM EDTA, 120 mM KCl, 10% glycerol, pH 7.4) and used as the source of receptors. All procedures were conducted at 4°C. Membranes were stored at −20°C and stable for at least 2 months. Protein concentration was determined by Bradford method.

PHOSPHORYLATION ASSAYS

Rat fetal membranes (400–600 μg protein) were stimulated in the presence of Ang II alone or in combination with different Ang II competitors at the indicated concentrations. Controls were done replacing Ang II with water. When indicated, Na_3VO_4 (1 mM) was included in the assay, 10 min prior to stimulation. The reaction was performed in a final volume of 100 μl for the indicated times at 20°C in phosphorylation buffer (20 mM HEPES, 60 mM NaCl, 0.1% BSA, pH 7.2). After stimulation, phosphorylation was conducted in the presence of ATP-Mg for 10 min (0.1 mM ATP, 20 mM $MgCl_2$). The reaction was stopped by adding 1 ml of cold phosphorylation buffer. The membrane proteins were recovered by centrifugation at 15,000*g*, solubilized and immunoprecipitated.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

After phosphorylation assays, pellets were solubilized in buffer A (20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, pH 7.2) containing 1.2% of CHAPS or 1% of Triton X-100 (60 min at 4°C) with gentle agitation and then centrifuged at 15,000*g* for 30 min. Immunoprecipitations were carried out by adding to the supernatants the indicated primary antibodies (2 μl). Antibodies were allowed to equilibrate with solubilized proteins overnight at 4°C, followed by the addition of protein A/G plus agarose (15 μl) for 3 h at 4°C. Immunocomplexes were recovered by centrifugation at 1,000*g*, washed 3 times with buffer A, and subjected to SDS–PAGE (7.5%). Proteins were transferred onto PVDF membranes and blots were blocked with 3% BSA in TBS (20 mM Tris–HCl pH 7.4, 0.9% NaCl) for 60 min at room temperature.

Proteins were immunoblotted with the indicated antibodies (1:1,000) followed by incubation with the secondary antibody (1:2,000 for AP-coupled and 1:5,000 for HRP-coupled antibodies). Blots were developed with BCIP-NBT for Alkaline Phosphatase-coupled antibodies or ECL for HRP-coupled antibodies and the results were analyzed by the Adobe Photoshop Software. Stripping of membranes was achieved by incubation in buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol) for 10 min, 50°C. Membranes were reprobed for loading control.

BINDING ON IMMUNOCOMPLEXES

For binding studies on the immunocomplexes, rat fetal membranes were stimulated with Ang II (10^{-8} M) in the absence or in the presence of PD123319 (10^{-6} M), phosphorylated and solubilized. Binding was performed on CHAPS-solubilized rat fetal membranes by incubating with $[^{125}I]Sar^1Ile^8$Ang II 10 nM for 90 min at RT, either in the absence or in the presence of cold Ang II (10^{-6} M, non-specific binding) or PD123319 (10^{-6} M), followed by immunoprecipitation with anti-SHP-1 antibody and Protein A–agarose. Radioactivity kept on the immunocomplexes was measured in an LKB gamma counter.

IMMUNOPRECIPITATION AND SHP-1 ACTIVITY ASSAY

Following stimulation, the supernatants of Triton X-100 solubilized membranes (100–150 μg protein) were incubated overnight with 2 μl of anti-SHP-1 or anti-AT_2 antibodies and processed as before. The immunocomplexes were resuspended in 40 μl PTPase buffer (20 mM HEPES, 60 mM NaCl, 0.1% BSA, pH 7.2) and 40 μl of Tyr-phosphopeptide (DADE(pY)LIPQQG) were added at final concentration 10^{-4} M using PTPase assay kit (Promega) according to the manufacturer's protocol. The PTPase activity was assayed by measuring release of inorganic phosphate from phosphopeptides based on a malachite green detection system [Harder et al., 1994]. Following incubation for 30 min at RT, 400 μl of Dye mixture solution was added and optical densities measured at 600 nm by spectrophotometer and quantified using the calibration curve performed with standards (Promega). To test the participation of c-Src in SHP-1 activation via AT_2 receptors, membranes were pre-incubated with PP2 (10 μM, 1 h), an Src inhibitor or its inactive analog PP3 (10 μM, 1 h), as described [Bursell et al., 2007].

STATISTICAL ANALYSIS

Values are expressed as mean ± SEM in the text and figures. Western blots were quantified in Scion Image and analyzed using PRISM package. Values of P considered to be statistically significant: $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

ANG II INDUCES PROTEIN TYROSINE DEPHOSPHORYLATION

In a previous article we demonstrated that Ang II induced protein Tyr-dephosphorylation in rat fetal E20 membrane preparation [Ciuffo et al., 1998].

The present study aims at analyzing the molecular mechanisms involved in Ang II-mediated dephosphorylation. Initially, we performed time course experiments, in which rat fetal (E20) membranes (100 μg) were stimulated with Ang II (10^{-7} M) for different periods of time, and Tyr-phosphorylated proteins were detected by Western blot. Protein Tyr-dephosphorylation was maximal after 8 min of stimulation (Fig. 1). Ang II induced Tyr-dephosphorylation of proteins of different molecular masses (Fig. 1, arrows). Preincubation with sodium orthovanadate (1 mM, 10 min) leads to a large increase in phosphotyrosine-containing proteins at basal levels and prevents Ang II-induced dephosphorylation as previously described [Alvarez et al., 2003].

AT_2 RECEPTORS INDUCE TYR-PHOSPHORYLATION OF SHP-1 PTPase

The rapid and transient protein tyrosine dephosphorylation induced by Ang II and revised by vanadate, suggests the involvement of a vanadate-sensitive PTPase. Because SHP-1 was shown to be important for AT_2 signaling in different cell lines, we sought to determine its involvement in this pathway. To test this hypothesis, rat fetal membranes were stimulated with Ang II (10^{-7} M) and Tyr-phosphorylation of SHP-1 was analyzed. Proteins were solubilized and immunoprecipitated with anti-SHP-1 antibodies and the immunocomplexes were separated by SDS–PAGE. Phosphorylation of the PTPase was assessed by Western blotting, using mouse monoclonal PY99 anti-p-Tyr antibodies. Ang II induced a rapid and transient phosphorylation of SHP-1 (Fig. 2A), maximal after 1 min stimulation ($n = 6$). Blots were stripped and reprobed with anti-SHP-1 antibody and the ratio of SHP-1 phosphorylation/SHP-1 was plotted (Fig. 2A), lower panel.

To further characterize this effect, variable Ang II concentrations (10^{-6}–10^{-8} M) were assayed after 1 min stimulation. Even at low

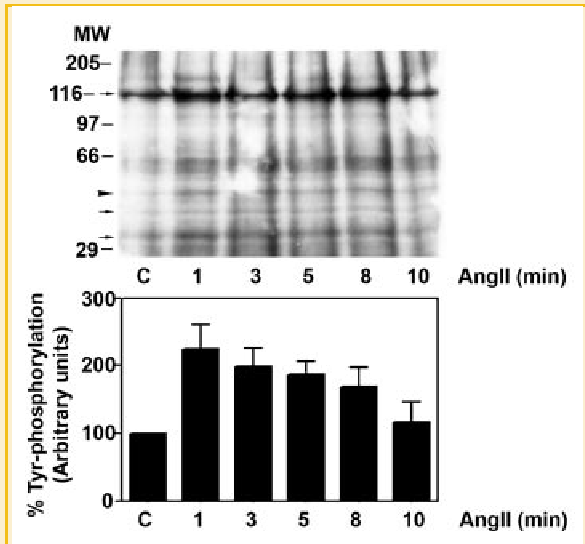

Fig. 1. Dephosphorylation induced by Ang II in rat fetal membranes. Rat fetal membranes (100 μg prot), were stimulated with Ang II (10^{-7} M) for different times. Proteins were subjected to 7.5% SDS–PAGE and immunoblotted with anti-p-Tyr antibody (PY99). Representative image and histogram of densitometric data as mean ± SEM (n = 3). Arrows point to the proteins analyzed.

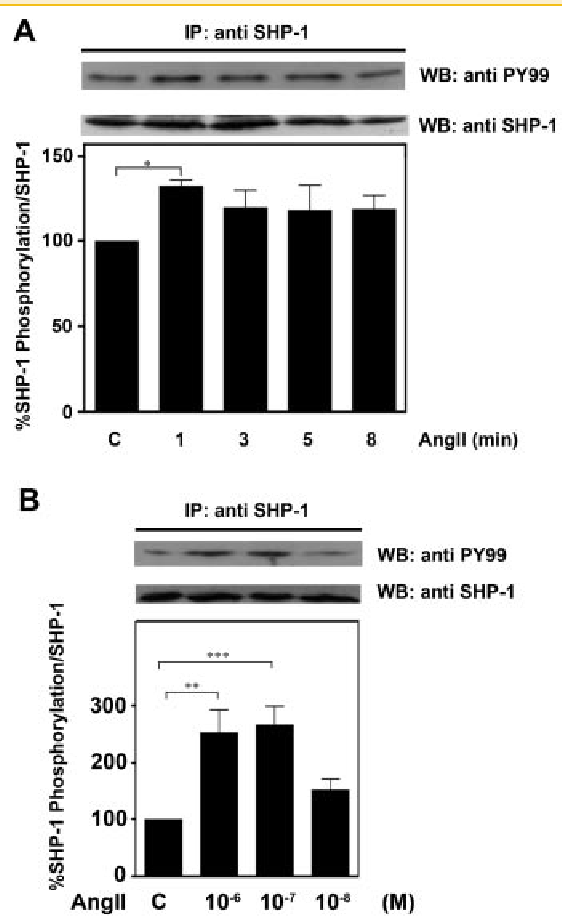

Fig. 2. SHP-1 Tyr-phosphorylation dependence of time and dose of Ang II. A: Time course of SHP-1 Tyr-phosphorylation. Membranes were stimulated with Ang II (10^{-7} M) for different times (1–8 min). C: Control without stimulation. Solubilized samples were immunoprecipitated with anti-SHP-1 antibody and then blotted with anti-p-Tyr antibody (upper panel). Blots were stripped and reprobed with anti-SHP-1 antibody (bottom). Representative image and histogram of the ratio of SHP-1 phosphorylation level/SHP-1 (mean ± SEM, n = 6). B: Dose-dependency of SHP-1 Tyr-phosphorylation. Membranes were stimulated for 1 min in the absence (C) or in the presence of Ang II (10^{-6}–10^{-8} M). Samples were solubilized, immunoprecipitated with anti-SHP-1 antibody and then blotted with either anti-p-Tyr antibody (upper panel). Blots were stripped and reprobed with anti-SHP-1 antibody (bottom). Representative image and histogram of the ratio of SHP-1 phosphorylation level/SHP-1 (mean ± SEM, n = 8).

Ang II concentrations (10^{-8} M), SHP-1 was phosphorylated (Fig. 2B). The time- and dose-dependent phosphorylation of SHP-1 suggest a biological meaning of the signal transduction studied.

Both Ang II receptor subtypes are present in E20 fetal membrane preparation. To identify the receptor subtype involved in SHP-1 phosphorylation, assays were performed in the presence of specific competitors for either AT_1 (Losartan) or AT_2 (PD123319) receptors. Aliquots of membrane preparation were preincubated (10 min) with different concentrations of the competitors (Losartan 10^{-6}–10^{-5} M, PD123319 10^{-6}–10^{-5} M), followed by stimulation with Ang II (10^{-7} M, 1 min) and phosphorylation assay. Competitors were present during Ang II stimulation. While the AT_1 competitor, Losartan, did not prevent SHP-1 Tyr-phosphorylation, the AT_2 antagonist, PD123319 completely blocked the effect of Ang II (Fig. 3), thus indicating that SHP-1 phosphorylation was mediated exclusively by AT_2 receptors. Blots were stripped and reprobed with anti-SHP-1 antibody and the ratio of SHP-1 phosphorylation/SHP-1 was plotted (Fig. 3).

AT_2 RECEPTORS ASSOCIATE TO SHP-1

To determine whether Ang II AT_2 receptors were physically associated to the phosphatase, co-immunoprecipitation assays were performed. After stimulation with Ang II (10^{-7} M) and phosphorylation, membranes were solubilized and immunoprecipitated with goat-anti AT_2 receptor antibody. Immunocomplexes were separated by SDS–PAGE and blotted with anti-SHP-1 antibody, revealing the presence of a polypeptide of apparent molecular weight 65 kDa corresponding to SHP-1 (Fig. 4A, upper panel). Equal protein loading was assessed by reprobing the membrane with anti-AT_2 antibody (Fig. 4A, lower panel). Co-immunoprecipitation of AT_2 receptors and SHP-1 phosphatase was inhibited by preincubation with the AT_2 competitor PD123319 (10^{-6} M) but not Losartan (10^{-6} M) (Fig. 4A). While both SHP-1 and AT_1 receptors were present in membranes (Fig. 4B, left panel), immunocomplexes obtained by using anti-AT_1 antibody did not contain SHP-1 (Fig. 4B, right panel). Taken together, these results indicate that SHP-1 associates to AT_2 but not to AT_1 receptors, following stimulation with Ang II (10^{-7} M).

To further confirm whether AT_2 receptors are coupled to SHP-1, we performed binding assays on CHAPS-solubilized membranes followed by immunoprecipitation with anti-SHP-1 antibodies.

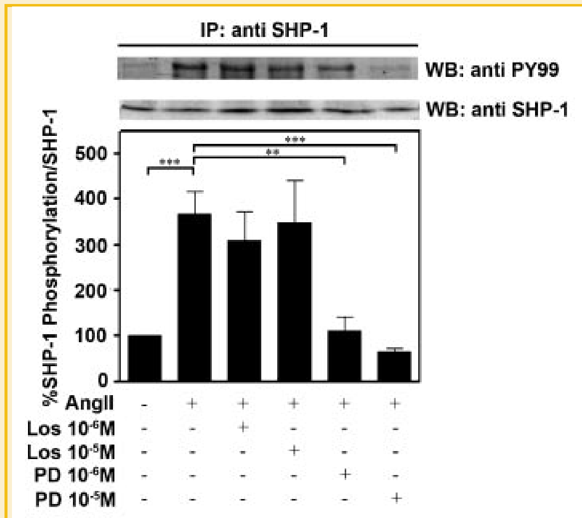

Fig. 3. SHP-1 Tyr-phosphorylation is mediated by Ang II AT_2 receptors. Membranes were stimulated with Ang II (10^{-7} M) for 1 min, either alone or plus Losartan (10^{-6}, 10^{-5} M), or PD123319 (10^{-6}, 10^{-5} M). Subsequently, membranes were solubilized, immunoprecipitated with anti-SHP-1 antibody and then blotted with anti-p-Tyr antibody (upper). Blots were stripped and reprobed with anti-SHP-1 antibody (bottom). Representative image and histogram of the ratio of SHP-1 phosphorylation level/SHP-1 (mean ± SEM, n = 8).

In order to promote SHP-1 association to AT_2 receptors, rat fetal membranes (600 μg protein) were stimulated with Ang II (10^{-8} M, 1 min) alone or in combination with PD123319 (10^{-6} M), followed by solubilization with CHAPS (1.2%). CHAPS-solubilized proteins were incubated at RT for 90 min with [^{125}I]Sar^{1}Ile^{8}Ang II (10 nM) either in the absence (total binding) or in the presence of an excess of Ang II (10^{-6} M, non-specific) or PD123319 (10^{-6} M, PD) (Fig. 4C). At the end of the assay, proteins were immunoprecipitated with anti-SHP-1 antibody and pellets were counted on an LKB gamma counter (Fig. 4C). The presence of PD123319 during the phosphorylation and binding assays prevented SHP-1 association to the receptor and thus ligand binding to AT_2 receptors. Non-specific binding, defined with an excess of Ang II (10^{-6} M), was comparable to binding in the presence of PD123319, thus suggesting the absence of AT_1 receptors in the immunocomplexes. Figure 4C shows the crude values of binding. Although Ang II concentration used during the stimulation step was low (10^{-8} M), we observed an induction of the association of SHP-1 to AT_2 receptors.

SHP-1 ACTIVITY IN AT_2 IMMUNOCOMPLEXES

The data shown in Figure 4 demonstrated the physical association of SHP-1 and AT_2 receptors, while Figure 2 shows the transient phosphorylation of SHP-1. To test if SHP-1 was activated after Tyr-phosphorylation, we performed PTPase activity assays on immunocomplexes obtained with anti-AT_2 antibodies in the presence or absence of PD123319 (10^{-6} M) after 1 min stimulation with Ang II (Fig. 5A). In both cases, Ang II enhanced the enzymatic activity of SHP-1, which was inhibited by preincubation with

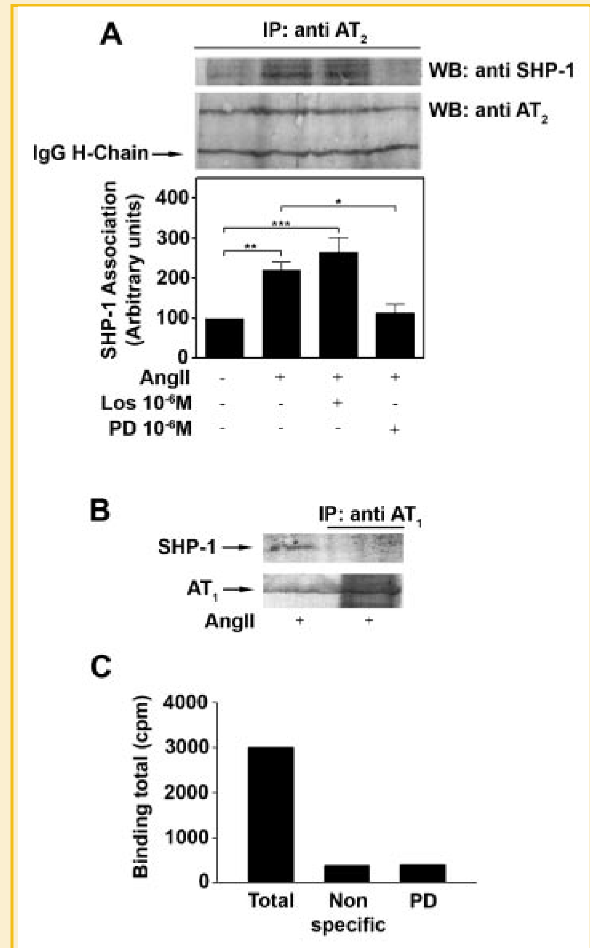

Fig. 4. SHP-1 Tyrosine phosphatase is associated to AT_2 receptors. A: Coprecipitation of SHP-1 and AT_2 receptor. Rat fetal (E20) membranes were stimulated for 1 min with Ang II (10^{-7} M), in the absence, or in the presence of Losartan (10^{-6} M), Los, or PD123319 (10^{-6} M, PD). Solubilized membranes were immunoprecipitated with goat anti-AT_2 antibody and developed with mouse anti-SHP-1 antibody (upper panel). Blots were stripped and reprobed with anti-AT_2 antibody (lower panel) to confirm equal precipitation across all lanes. Representative image and histogram of densitometric data as mean ± SEM (n = 5). B: Immunocomplexes obtained with anti-AT_1 antibody do not contain SHP-1. Lane 1: Rat fetal membranes were stimulated with Ang II (10^{-7} M, 1 min) and immunoblotted with anti-SHP-1 (upper panel) or anti-AT_1 (lower panel) antibody as a control. Lane 2: Rat fetal (E20) membranes were stimulated with Ang II (10^{-7} M, 1 min), solubilized and immunoprecipitated with anti-AT_1 antibody and developed with anti-SHP-1 antibody (upper panel) or with anti-AT_1 antibody (lower panel). C: AT_2 receptors are present on SHP-1 immunocomplexes. Binding of [^{125}I]Sar^{1}Ile^{8}Ang II (10 nM) was performed on CHAPS-solubilized membranes after Ang II stimulation. Rat fetal membranes (600 μg protein) were stimulated with Ang II (10^{-8} M, 1 min) alone or in combination with PD123319 (10^{-6} M, PD). CHAPS-solubilized proteins were incubated at RT for 90 min with [^{125}I]Sar^{1}Ile^{8}Ang II (10 nM) either in the absence total binding or in the presence of an excess of Ang II (10^{-6} M, non-specific) or PD123319 (10^{-6} M, PD). Proteins were immunoprecipitated with anti-SHP-1 antibody and pellets were counted on an LKB gamma counter.

the AT_2 competitor, PD123319 (Fig. 5A). Taken together, these results as well as the association between AT_2 and SHP-1 (Fig. 4), indicate that both, association and activation of SHP-1, result from the specific stimulation of AT_2 receptors by Ang II.

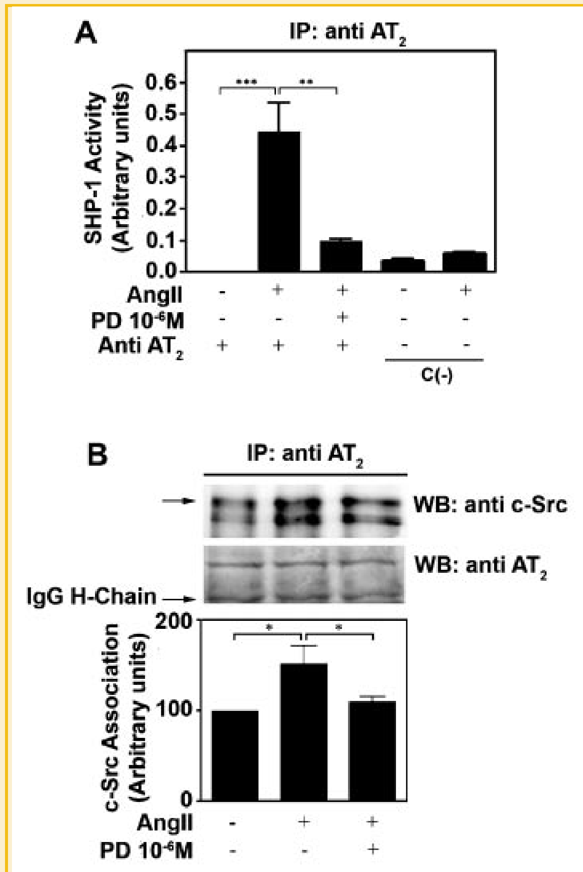

Fig. 5. SHP-1 activity in AT_2 immunocomplexes. A: Detection of SHP-1 activity in immunocomplexes obtained with anti-AT_2 antibody. Aliquots of membranes (600 μg) were stimulated with Ang II (10^{-7} M, 1 min) in the presence or absence of PD123319 (10^{-6} M), solubilized and immunoprecipitated. SHP-1 activity in immunocomplexes was determined by measuring the released phosphate (mean ± SEM, n = 3). C(-): IP control experiment without anti-AT_2 antibody. B: c-Src associates to AT_2 receptors. Co-immunoprecipitation of c-Src and AT_2 receptors. Rat fetal (E20) membranes were stimulated with Ang II (10^{-7} M, 1 min) in the presence or absence of PD123319 (10^{-6} M). Solubilized membranes were immunoprecipitated with anti AT_2 antibody and developed with anti-c-Src antibody (upper panel). Blots were stripped and reprobed with anti-AT_2 antibody (lower panel) to confirm equal precipitation across all lanes. Representative image and histogram of densitometric data as mean ± SEM (n = 8).

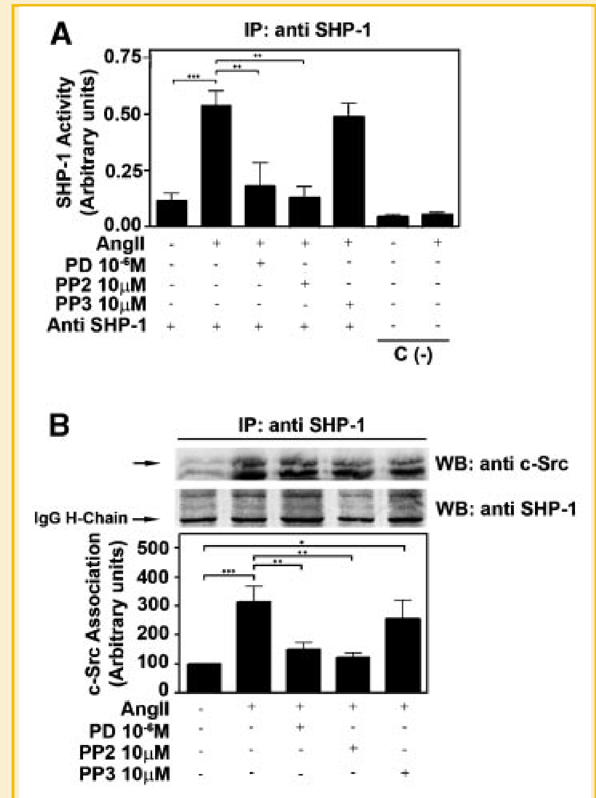

Fig. 6. c-Src is involved in SHP-1 activation by AT_2 receptors. A: Detection of SHP-1 activity in immunocomplexes obtained with anti-SHP-1 antibody. Aliquots of membranes (600 μg) were pre-incubated with PD123319 (10^{-6} M, 10 min), PP2 (10 μM, 60 min) or PP3 (10 μM, 60 min) and then stimulated with Ang II (10^{-7} M, 1 min). Membranes were solubilized, immunoprecipitated and SHP-1 activity in immunocomplexes was determined by measuring the released phosphate (mean ± SEM, n = 7). C(-): IP control experiment without anti-AT_2 antibody. B: c-Src associates to SHP-1 immunocomplexes. Following the assays shown in (A), immunocomplexes were recovered by centrifugation, washed and immunobloted to detect the presence of c-Src (upper panel) or SHP-1 (lower panel). Representative image and histogram of densitometric data as mean ± SEM (n = 8).

c-Src IS INVOLVED IN SHP-1 ACTIVATION BY AT_2 RECEPTORS

Tyrosine phosphorylation of SHP-1, following Ang II stimulation, suggested the contribution of a tyrosine kinase in this signaling pathway. To test this hypothesis, we searched for the recruitment of c-Src kinase family in AT_2 immunocomplexes. Figure 5B shows the presence of c-Src kinase in AT_2 immunocomplexes after 1 min stimulation with Ang II. Preincubation with PD123319 (10^{-6} M) prevented c-Src recruitment.

To confirm the previous results we measured SHP-1 activity in SHP-1 immunocomplexes. Ang II (10^{-7} M, 1 min) induced SHP-1 activity, while an excess of the AT_2 competitor PD123319 prevented SHP-1 activation (see Fig. 6A). To test the participation of c-Src in SHP-1 activation via AT_2 receptors, we performed the assay in the presence of c-Src inhibitor PP2 or its inactive analog PP3. Preincubation with PP2 (10 μM, 60 min) prevented SHP-1 activation (Fig. 6A) while the activity in presence of PP3 (10 μM, 60 min) was unchanged.

To further verify c-Src participation in this pathway, we searched for the presence of c-Src kinase in SHP-1 immunocomplexes, recovered from the activation assay. Preincubation with PP2 (10 μM, 60 min) prevented SHP-1 activation as well as c-Src association to SHP-1 immunocomplexes (Fig. 6B).

From the results shown in Figures 5 and 6, activation of AT_2 receptors induce the formation of a complex, where c-Src or a member of the non-receptor tyrosine kinase family, mediate the phosphorylation and activation of SHP-1. Thus, SHP-1 mediates protein Tyr-dephosphorylation, leading to specific cell effects.

DISCUSSION

The aim of the present study was to analyze the regulation of SHP-1 phosphatase activity following Ang II receptor stimulation and to investigate the coupling of AT_2 receptors with PTPase in membrane preparations from rat fetal (E20) tissues. Fetal membranes were selected as a model of study because fetuses represent a physiological developmental state where both Ang II receptor subtypes are expressed and because it has been postulated that Ang II receptors could be involved in growth modulation. Moreover, opposite intracellular effects of AT_1 and AT_2 receptors on vasoconstriction/vasodilation and proliferation/apoptosis, among others, have been shown [Inagami et al., 1999; Horiuchi et al., 1999; Landon and Inagami, 2005; Deshayes and Nahmias, 2005].

In a previous article, we demonstrated that Ang II receptors mediate Tyr-dephosphorylation of several proteins in fetal E20 membrane preparations. We left an open question whether Ang II stimulates a Tyr-phosphatase or inhibits a Tyr-kinase. In the present article, we address this question and demonstrate, in membrane preparations from fetal (E20) rats which contain both Ang II receptor subtypes, the involvement of c-Src in the activation of PTPase SHP-1, mediated by Ang II AT_2 receptors.

Although discrepancies exist regarding the role of G-proteins in the AT_2 pathway, it is now accepted that AT_2 receptors can couple to multiple signaling molecules. However, there is consistency among different authors that stimulation of AT_2 receptors leads to activation of tyrosine or serine/threonine phosphatases in different cell lines and fetal tissues [Bousquet et al., 1998; Cui et al., 2001; Gendron et al., 2003; Nouet et al., 2004; Wu et al., 2004; Li et al., 2007].

By means of immunoprecipitation studies combined with Western blot we demonstrated that Ang II elicits a dose-dependent, rapid and transient phosphorylation of SHP-1 phosphatase. Competition studies with specific antagonists showed that only AT_2 receptors are involved in the phosphorylation of SHP-1. The rapid time-course of SHP-1 phosphorylation (maximal at 1 min) precedes the induction of protein tyrosine dephosphorylation (maximal at 8 min), suggesting that SHP-1 is an upstream component in the AT_2 intracellular cascade. The lag time between maximal SHP-1 phosphorylation and maximal protein tyrosine dephosphorylation suggest that SHP-1 affects downstream substrates. Although SHP-1 regulation seems to be highly complex, our observations agree with previous reports from several authors [Cui et al., 2001; Matsubara et al., 2001; Feng et al., 2002; Frank et al., 2004] where activation of SHP-1 was associated to tyrosine phosphorylation.

Co-precipitation studies demonstrated that SHP-1 physically associates to AT_2 receptors. The physical association was induced by receptor stimulation and selectively blocked by PD123319, thus confirming the involvement of AT_2 receptors and suggesting an active role for these receptors. Miura and Karnik [2000] and Miura et al. [2005] proposed that AT_2 receptors are constitutively activated on cells over expressing AT_2 receptors. We assume that transfected cells may differ from the physiological situation. In contrast, our model of rat fetal (E20) membrane preparations represents a physiological state where both receptor subtypes coexist [Ciuffo et al., 1998].

SHP-1 activity was detected in immunocomplexes, evidencing an increased activity following Ang II stimulation, which was selectively blocked by PD123319. This effect, observed in both anti-AT_2 and anti-SHP-1 immunocomplexes demonstrates that Tyr-phosphorylation of SHP-1 leads to activation of the phosphatase, as previously suggested [Feng et al., 2002]. On the other hand, we do not observe a constitutive physical association between the AT_2 receptor and SHP-1, as was indicated by other authors in an enforced expression model [Feng et al., 2002; Miura et al., 2005]. Over-expression of proteins can lead to effects that differ from those present in vivo. Our model, which takes ex vivo material at a time where high levels of AT_2 receptors are expressed, might represent a physiological state during development. Recent studies have been conducted on tissues where both Ang II receptor subtypes are expressed, such as fetal VSMC cells and fetal brain, where AT_2 receptors seem to mediate cell differentiation with participation of SHP-1 [Wu et al., 2004; Li et al., 2007].

AT_2 receptors belong to the superfamily of G-protein coupled receptors with seven transmembrane domains and therefore, they lack intrinsic catalytic activity. Since several authors pointed out that c-Src can modulate SHP-1 activity [Somani et al., 1997; Frank et al., 2004], we looked for the presence of c-Src in the immunocomplexes. Interestingly, following receptor stimulation (1 min) c-Src was associated to AT_2 or SHP-1 immunocomplexes. Precinubation with the c-Src inhibitor PP2 inhibited SHP-1 activation and c-Src association, thus confirming the participation of c-Src in this pathway.

Development is an intrincated process where growth and anti-growth signals combine in a coordinated way and protein phosphorylation is part of the process control. A number of recent evidences suggest opposite effects of Ang II receptor subtypes AT_1 and AT_2 [Inagami et al., 1999; Horiuchi et al., 1999; Landon and Inagami, 2005; Deshayes and Nahmias, 2005]. Although the role of the AT_2 receptor in fetal development is not yet clearly established, the fact that they are coupled to SHP-1 phosphatase in fetal tissues during a critical developmental stage, is a strong support for the anti-growth role of AT_2 receptors. Both AT_1 and AT_2 receptors in rat fetal membrane preparations may play a crucial role in determining cell fate during developmental stages by mutual interactions.

In summary, we demonstrate here for the first time the involvement of c-Src in SHP-1 activation via AT_2 receptors present in an ex vivo model expressing both receptor subtypes. In this model, AT_2 receptors are not constitutively associated to SHP-1 nor is SHP-1 constitutively activated. Thus, we clearly establish that AT_2 receptors mediate tyrosine phosphorylation and activation of SHP-1, leading to protein tyrosine dephosphorylation in rat fetal membranes, by a mechanism involving c-Src kinase association and activation.

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