Pivotal role of tyrosine phosphatase SHP-1 in AT2 receptor-mediated apoptosis in rat fetal vascular smooth muscle cell

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Abstract

Objective: To examine the possible crosstalk and the roles of angiotensin (Ang) II type 1 (AT1) and type 2 (AT2) receptors in the control of apoptosis in fetal vascular smooth muscle cells (VSMCs). Methods: Fetal VSMCs were prepared from rat fetal aorta at embryonic day 20. Expression of Ang II receptors was measured by a radioligand binding assay. Apoptotic changes were assessed by caspase 3 activity and chromatin dye staining. Regulation of extracellular signal-regulated kinase (ERK) activity via Ang II receptors was analysed by determining phosphorylated ERK with Western blot. Ang II receptor-mediated activation of tyrosine phosphatase SHP-1 was assessed by protein tyrosine phosphatase assay. Results: The expression of AT1 and AT2 receptors was approximately 70%: 30% per cell. Serum depletion induced apoptosis in fetal VSMCs and selective AT1 receptor stimulation attenuated the apoptotic changes, whereas selective AT2 receptor activation enhanced apoptosis. Ang II increased ERK phosphorylation, which was inhibited by addition of the AT1 receptor-specific antagonist CV11974, but enhanced by addition of the AT2 receptor-specific antagonist PD123319, suggesting that activation of AT2 receptor attenuated the AT1 receptor-mediated ERK phosphorylation. Moreover, we demonstrated that AT2 receptor stimulation activated SHP-1 in fetal VSMCs, whereas AT1 receptor stimulation did not. Transient transfection of a dominant-negative SHP-1 mutant into rat fetal VSMCs resulted in a significant decrease of the AT2 receptor-mediated inhibition of ERK phosphorylation and attenuated the proapoptotic effect of AT2 receptor. Conclusion: These results indicate that a crosstalk between AT1 and AT2 receptors regulates the survival of fetal VSMCs and substantiate SHP-1 as a key molecule in AT2 receptor signaling.

Keywords: Angiotensin; Apoptosis; Receptor; Signal transduction; Smooth muscle

1. Introduction

Angiotensin (Ang) II is classically considered as a vasoconstricting, progrowth molecule acting via stimulation of the G protein-coupled seven membrane-spanning Ang II type 1 (AT1) receptor. In addition to the ‘classical’ AT1 receptor, it is now conceivable that Ang II activates another receptor subtype (type 2 or AT2 receptor). Although both AT1 and AT2 receptors belong to the seven-transmembrane, G protein-coupled receptor family, recent evidence reveals that the functions of AT1 and AT2 receptors are mutually antagonistic. The AT2 receptor is abundantly and widely expressed in fetal tissues, but present only at low levels in adult tissues and re-expressed in certain pathological conditions such as ovarian atresia, wound injury, myocardial infarction, and vascular injury [1]. The highly abundant expression of this receptor during embryonic and neonatal growth and its quick disappearance after birth has led us to the suggestion that this receptor may be involved in growth, development and/or
differentiation. In many organs, tissue remodeling and
degeneration involve an active process, termed apoptosis
(programmed cell death), which is a ubiquitous, evolution-
ally conserved, physiological mechanism of cell death that
regulates mass and architecture in many tissues. The
genetic program for apoptosis may be activated at a
defined time during embryogenesis or in the maturation of
adult cells, by a wide range of physiological stimuli. These
results provide us with the possibility that AT2 may induce
apoptosis. Actually proapoptotic effects of AT2 receptor
have been demonstrated in PC12W cells [2–5], R3T3
mouse fibroblasts [2,4], rat ovarian granulosa cells [6],
human umbilical venous endothelial cells [7] and AT2
receptor-transfected adult rat VSMCs [8].

The growth inhibitory effects of the AT2 receptor have
been shown to associate with the activation of a series of
phosphatases including the protein tyrosine phosphatase
SHP-1 [9,10] or the mitogen-activated protein kinase
phosphatase-1 (MKP-1) [2,4], and the serine/threonine
phosphatase 2A (PP2A) [11], which results in the inactivation
of AT1 receptor- and/or growth factor-activated
extracellular signal-regulated kinase (ERK). SHP-1 [for-
termed PTP1C, SH-PTP-1, SHP, and HCP], a two Src
homology 2 (SH2) domain-containing cytosolic
tyrosine phosphatase, has been implicated in the negative
regulation of a broad spectrum of growth-promoting
receptors, including receptor tyrosine kinases such as c-Kit
[12,13], Trk A [14], and epidermal growth factor (EGF)
[15] receptors; cytokine receptors such as interleukin-3
(IL-3) [12], interferon-α/β [16] and erythropoietin [17]
receptors; and B cell FcγRIIB [18] receptor. Given the
breadth of its interaction with multiple growth factor
receptors, we hypothesize that SHP-1 is involved in AT2
receptor intracellular pathway which negatively crosstalks
with AT1 receptor signaling. Moreover, we have recently
observed that SHP-1 is one of the most proximal effector
in AT2 receptor-mediated ERK inhibition and apoptosis in
PC12 cells [10].

Accordingly, we used fetal VSMC, which endogenously
express both AT1 and AT2 receptors, and examined the
possibilities that AT2 receptor stimulation exerts proapoptotic
effects by antagonizing the function of AT1 receptor
and that SHP-1 is pivotal in the proapoptotic effect of AT2
receptor stimulation.

2. Methods

The investigation conforms with the Guide for the Care
and Use of Laboratory Animals published by the US
National Institutes of Health (NIH Publication No. 85-23,
revised 1996).

2.1. Cell culture

Fetal VSMCs were prepared from the thoracic aortae of
Sprague–Dawley rat fetuses (embryonic day 20) and
cultured in Dulbecco’s modified Eagle’s medium (DMEM)
(Life Technologies Inc., Rockville, Maryland, USA) sup-
plemented with 10% fetal bovine serum (FBS) as previ-
ously described [19]. In addition to the morphological
observation, characterization of VSMC was examined by
indirect immunofluorescence using a monoclonal antibody
specific for α-smooth muscle actin (clone 1A4; Sigma
Chemical Co.) [20]. Our culture of fetal VSMCs exhibited
the ‘hill-and-valley’ appearance which is characteristic of
adult VSMC. The cells were uniformly positive for α-
smooth muscle actin by immunofluorescence. These results
indicate that the cells are characteristic of VSMC although
the possible contamination of fibroblasts and endothelial
cells cannot be avoided. All the experiments were per-
formed using the cells at the second or third passage. AT1
and AT2 receptor binding was measured as previously
described [19].

2.2. Plasmid constructs and transfection

The SHP-1 (C453/S) mutant and SHP-2 (C463/S)
mutant cDNA were inserted into the pcDNA3 vector [21].
Transient transfection was performed with 0.5 μg of
plasmid DNA per well of a six-well plate and Lipofec-
tAMINE PLUS (GIBCO-BRL) according to the manufac-
turer’s instructions (DNA:LipofectAMINE:PLUS ratio,
1:10:10 μl). After transient transfection, the transfected
cells were fed with complete growth medium for 48 h to
allow protein expression. Transfected cells were identified
by β-galactosidase staining. The transfection efficiency
was 25±5%, a similar level as previously reported [22].
Overexpression of SHP-1 (C453/S) mutant and SHP-2
(C463/S) mutant was confirmed by immunoblot with an
anti-SHP-1 or an anti-SH-2 antibody (Santa Cruz Bio-
technology, Inc.).

2.3. Determination of cell death

Rat fetal VSMCs were grown in 10% FBS DMEM to a
subconfluent state in 10-cm dishes and incubated for an
additional 48 h in serum-free DMEM. Nuclear chromatin
morphology analysis and caspase-3 activity assay were
used as quantitative indices of apoptosis. Nuclear chro-
matin morphology was analyzed by staining with Hoechst
33342 (Molecular Probes, Inc., Eugene, Oregon, USA) and
viewed under fluorescence microscopy, as previously
described [2]. The caspase-3 activity assay was performed
using an adaptation of a previously described protocol
[23]. Results were normalized to basal activity in viable
cells maintained in serum-containing medium.

2.4. Determination of ERK activity

Subconfluent VSMCs were incubated in serum-free
DMEM for 24 h and were treated with vehicle or Ang II

Determination of ERK activity

Subconfluent VSMCs were incubated in serum-free
DMEM for 24 h and were treated with vehicle or Ang II
and National Institutes of Health image software. Densities was performed using an image scanner (EPSON GT-8000)3.2. chemiluminescence (Amersham). Densitometric analysis was performed using an image scanner (EPSON GT-8000) and National Institutes of Health image software. Densities of the bands corresponding to ERK-1/2 were added to represent total ERK.

2.5. Measurement of SHP-1 tyrosine phosphatase activity

Subconfluent VSMCs were incubated in serum-free DMEM for 24 h and were treated with vehicle or Ang II (0.3 μmol/l) with or without PD123319 (10 μmol/l) or CV11974 (10 μmol/l) for 3 min. SHP-1 tyrosine phosphatase activity was measured as previously described [9,10].

2.6. Data analysis

All values are expressed as mean±S.E.M. The data were evaluated by analysis of variance followed by a Newman–Keuls’ test for multiple comparisons. The difference was considered to be significant if P<0.05.

3. Results

3.1. Cross-talk of AT1 and AT2 receptors regulate cell death in fetal VSMCs

To examine the role of endogenous AT2 receptor in Ang II regulated apoptosis, we employed VSMC prepared from rat fetal aorta. VSMCs were cultured from the aorta of fetal rat at embryonic day 20, and shown to express both AT1 and AT2 receptors (AT1 receptor 11.7±0.65 fmol/10⁶ cells; AT2 receptor, 5.27±2.28 fmol/10⁶ cells, n=6, mean±S.E.). Upon serum starvation for 24 h, chromatin dye staining showed that VSMCs underwent morphological changes of typical apoptosis, such as fragmented nuclei with condensed chromatin (data not shown). We characterized the time course of caspase-3 activation after serum deprivation in our system and we examined the effects of Ang II (Fig. 1A,B), since caspase-3 plays a central role in serum or trophic factor deprivation-induced apoptosis.

Serum starvation induced a significant increase in caspase-3 activity, reaching a maximum at 12 h and declining to basal level within 48 h (Fig. 1A). However, the addition of Ang II (0.3 μmol/l) significantly inhibited caspase-3 activation measured at 12 h after serum depletion (Fig. 1B). Stimulation with Ang II (0.3 μmol/l) in the presence of AT2 receptor-specific antagonist PD123319 (10 μmol/l) further inhibited caspase-3 activity compared with stimulation with Ang II alone (Fig. 1B). On the contrary, addition of the AT1 receptor-specific antagonist CV11974 (10 μmol/l), Ang II stimulation further increased the serum starvation-induced caspase-3 activation (Fig. 1B). Neither CV11974 nor PD123319 alone influenced the caspase 3 activity (Fig. 1B).

3.2. Ang II activates ERK via the AT1 receptor and inhibits ERK via the AT2 receptor in fetal VSMCs

To explore the molecular mechanism involved in the cross-talk of AT1 and AT2 receptors in regulating cell death in fetal VSMCs, we examined ERK activation in response to the stimulation of AT1 or/and AT2 receptors. As shown in Fig. 2A and B, immunoblot using an anti-phospho-ERK antibody demonstrates that Ang II (0.3 μmol/l) stimulation significantly increased ERK phosphorylation. CV11974 (10 μmol/l) strongly attenuated the Ang II-induced ERK phosphorylation. In contrast, PD123319 (10 μmol/l) significantly enhanced the Ang II-induced ERK phosphorylation. ERK phosphorylation was not affected by treatment with CV11974 (10 μmol/l) and PD123319 (10 μmol/l) alone.

3.3. Activation of AT2 receptor increases SHP-1 activity in fetal VSMCs

We first examined the protein level of SHP-1 in rat fetal VSMCs and found that SHP-1 is expressed in fetal VSMC (Fig. 3A). Next we examined the SHP-1 activity and observed that Ang II (0.3 μmol/l) stimulation for 3 min significantly increased SHP-1 activity and this Ang II-mediated SHP-1 activation was blocked with PD123319 (10 μmol/l) but not by CV11974 (10 μmol/l) (Fig. 3B).

3.4. Role of SHP-1 in AT2 receptor-induced apoptosis in fetal VSMCs

To investigate whether SHP-1 plays a role in the proapoptotic effect of AT2 receptor, we transfected rat fetal VSMCs with a dominant negative (dn) SHP-1 mutant in which the active site cysteine 453 was mutated to serine (C453/S) [21]. Overexpression of dnSHP-1 mutant was confirmed by immunoblot showing 6-fold increase in SHP-1 immunoreactivity compared with the control vector pcDNA3 transfected cells (Fig. 3A). Basal SHP-1 activity was low in VSMCs transfected with dnSHP-1 mutant and...
Fig. 1. Effect of the stimulation of AT1 receptor or AT2 receptor on apoptosis in rat fetal VSMCs. (A) Time course of caspase-3 activity in rat fetal VSMCs in response to serum deprivation. Subconfluent rat fetal VSMCs were maintained in serum-free DMEM for indicated times. Values are the mean±S.E.M. (n=6). *P<0.05 and **P<0.01 vs. control (0 h). (B) Effect of Ang II on caspase-3 activity. Subconfluent fetal VSMCs were maintained in serum-free DMEM and stimulated with Ang II (0.3 μmol/l) with or without CV11974 (10 μmol/l) or PD123319 (10 μmol/l) for 12 h. Values are the mean±S.E.M. (n=6). *P<0.05 and **P<0.01 vs. control (serum). ↑P<0.05 vs. serum free. S, serum; SF, serum free.

we could not observe further significant increase in SHP-1 activity in response to Ang II (Fig. 3C). We then examined the functional significance of SHP-1 activation on AT2 receptor-mediated ERK inhibition and apoptosis. Since the SHP-2 phosphatase [formerly termed SHPTP-2, SHPTP-3, Syp, PTP2C, and PTP1D], in contrast to SHP-1, frequently functions as a positive effector of growth-stimulatory signaling pathway, we also examined the possible involvement of SHP-2 in the regulation of ERK activation and cell death by Ang II in rat fetal VSMCs. After identification of endogenous expression of SHP-2 in rat fetal VSMCs by immunoblot, we transfected rat fetal VSMCs with a dnSHP-2 mutant in which the active site cysteine 463 was mutated to serine (C463/S). Overexpression of dnSHP-2 mutant was confirmed by immunoblot showing 3-fold increase in SHP-2 immunoreactivity compared with the control vector pcDNA3 transfected cells (Fig. 3A). We first examined the ERK activation in cells overexpressing dnSHP-1 or dnSHP-2 (Fig. 4). In dnSHP-1 transfected VSMCs, increase in ERK activity by Ang II was further significantly enhanced compared to that in pcDNA3 transfected VSMCs and ERK activation by Ang II was not influenced with PD123319 (Fig. 4). In contrast, dnSHP-2 transfection did not modify the effect of Ang II on ERK activation (Fig. 4). Moreover, we observed that transfection of dnSHP-1 or dnSHP-2 did not influence the protein levels of total ERK (Fig. 4A). Next we examined the effect of Ang II treatment on apoptotic changes in dnSHP-1 or dnSHP-2 transfected rat fetal VSMCs (Fig. 5). Upon serum starvation for 12 h, a near 4-fold increase in caspase-3 activity was observed in each transfectant (Fig. 5). In control vector pcDNA3 transfected VSMCs, Ang II (0.3 μmol/l) stimulation led to inhibition of the caspase-3 activation, which was enhanced by addition of PD123319 (10 μmol/l), and selective activation of AT2 receptor by Ang II in presence of CV11974 (10 μmol/l) further increased the caspase-3 activation induced by serum-starvation (Fig. 5), as that observed in non-transfected VSMCs (Fig. 1B). However, the enhancement of Ang II stimulation-induced inhibition of the caspase-3 activation by PD123319 or the aggravation of serum starvation-induced caspase-3 activation by Ang II in presence of CV11974 were blocked by overexpression of dnSHP-1 in VSMCs (Fig. 5). Transfection of dnSHP-2 did not modulate the effects of Ang II on caspase-3 activation caused by serum depletion in VSMCs (Fig. 5).
4. Discussion

Increasing evidence reveals that AT2 receptor acts as an antagonist against the classical AT1 receptor, i.e. the AT2 receptor exerts antigrowth, antihypertrophic, and proapoptotic effects and promotes differentiation [1]. Expression of the AT2 receptor in rat embryonic blood vessels is ‘turned on’ at late gestation (embryonic days 16–21) and early neonatal period, but decreases rapidly to very low levels in the adult vessel [24,25]. The spatial and temporal patterns of rat vascular AT2 receptor expression, together with its growth modulatory action, have led us to hypothesize that...
this receptor plays an important role in vascular development and remodeling in late gestation. Indeed, pharmacological blockade of the AT2 receptor using the specific antagonist PD123319 during rat embryonic days 16–21 results in increased aortic DNA synthesis [24]. Our previous results using AT2 receptor null mice suggest that AT2 receptor promotes vascular differentiation and contributes to vasculogenesis [26]. Moreover, we observed that VSMCs prepared from the aorta of fetal or newborn AT2 receptor null mice showed enhanced proliferative activity [19]. Here we demonstrate that Ang II regulates apoptosis in fetal VSMCs, i.e. stimulation of AT1 receptor inhibits apoptosis, whereas activation of AT2 receptor potentiates it. These results provide further support to the hypothesis that Ang II-regulated programmed cell death is involved in vascular development or vasculogenesis.

To explore the molecular mechanism of Ang II-regulated apoptosis in fetal VSMCs, we focused on ERK activation since the ERK cascade is crucial in proliferation, differentiation, and hypertrophy or apoptosis of VSMC [27], and is likely involved in AT2 receptor-mediated antigrowth and proapoptotic actions on VSMC [8,24,28]. In cultured VSMCs prepared from embryonic day 20 rat, we found that activation of AT1 increases ERK phosphorylation and activation of AT2 receptor shows an inhibitory effect on the AT1 receptor-mediated ERK phosphorylation. Such opposite modulation of ERK by AT1 and AT2 receptors was also observed in rat cultured neurons, which contained both AT1 and AT2 receptors [29]. Our results suggest that the facilitating effect of AT2 receptor stimulation on serum deprivation-induced cell death in fetal VSMCs is at least partly due to the inhibitory effect of AT2 receptor activation on ERK phosphorylation.

SHP-1 is a soluble tyrosine phosphatase that participates in the negative regulation of receptor tyrosine kinase pathway [12–15]. Our previous results have shown that stimulation of AT2 receptor rapidly activates SHP-1 in N1E-115 and AT2 receptor-transfected Chinese hamster ovary (CHO) cells [9], and that SHP-1 is a proximal effector of the AT2 receptor, implicated in the apoptotic effect of AT2 receptor in PC12 cells [10]. Therefore we speculated that SHP-1 may be a key molecule in AT2 receptor signaling in rat fetal VSMCs. We observed that stimulation of AT2 receptor also rapidly activates SHP-1 in rat fetal VSMCs, where AT1 activation does not. To establish a functional link between AT2 receptor-mediated...
activation of SHP-1 and inactivation of ERK, we used a catalytically inactive, dominant negative (dn) mutant SHP-1 and demonstrated that preventing SHP-1 activation abrogates AT2 receptor stimulation-induced ERK inhibition in rat fetal VSMCs. Moreover, the ERK inhibition is due to activation/phosphorylation, not to translation of ERK, since total protein level of ERK was not changed by overexpression of dnSHP-1 in rat fetal VSMCs.

It has already been demonstrated that for a number of membrane receptors such as EGF receptor, SHP-1 terminated these receptors’ signal by direct interaction with the receptors [12,15,17,18]. Tyrosine kinase activation by the AT1 receptor is at least partly attributed to transactivation of a tyrosine kinase receptor such as the epidermal growth factor (EGF) receptor or the platelet derived growth factor (PDGF) receptor [30–32]. In VSMC, Ang II stimulation has been shown to activate the EGF receptor by tyrosine phosphorylation [31]. Very recent evidence showed that activation of AT2 receptor results in functional transactivation of insulin receptor kinase in CHO cells, leading to inhibition of ERK phosphorylation and cell proliferation [33]. Therefore, it is likely that ERKs may not be direct cellular targets for AT2 receptor-activated SHP-1, but rather that AT2 receptor-activated SHP-1 affects upstream intermediates of AT1 receptor-induced ERK pathway such as EGF receptor.

The result of inhibition of AT2 receptor-mediated apoptosis by overexpression of dnSHP-1 suggests that the proapoptotic effect of AT2 receptor is associated with the inactivation of ERK via a signaling pathway involving the activation of SHP-1 in rat fetal VSMCs. In addition, sst2 somatostatin receptor-mediated activation of SHP-1 has been shown to act as an initial transducer of the antiproliferating signaling mediated by this receptor [21]. It is interesting that SHP-1 has been reported to be required for the delivery of the Fas apoptosis signal in lymphoid cell [34]. These observation suggest that SHP-1 is a transducer of the antimitogenic and/or proapoptotic signals mediated.
which contributes to the pathogenesis and remodeling of cardiovascular diseases.

In summary, these results provide us with direct evidences that crosstalk between AT1 and AT2 receptor controls the fate of fetal VSMCs. It is also possible that this antagonistic interaction may contribute to the fetal vascular development or vasculogenesis. We also demonstrated that SHP-1 is a pivotal effector in the signal transduction pathway of AT2 receptor in fetal VSMCs.

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References

[7] Dimmeler S, Rippmann V, Weiland U, Haendeler J, Zeiher AM. SHP-2 functions as an adaptor protein for Janus kinase 2 (JAK2) association with AT1 receptor, thereby facilitating JAK2 phosphorylation and activation [38]. However, our data showed that preventing SHP-2 activation with transfection of dnSHP-2 into fetal VSMCs did not affect either AT1 receptor-mediated ERK phosphorylation and cell survival or AT2 receptor-mediated ERK inhibition and cell death. These results indicate that SHP-2 may not be involved in the crosstalk between AT1 and AT2 receptors in regulating ERK activation and cell death in fetal VSMCs.

The results of this study may be relevant to vascular pathobiology. It has been shown that neointimal VSMCs in vascular lesions such as atherosclerosis and restenosis after balloon angioplasty resemble fetal VSMCs, rather than adult medial VSMCs [39,40]. AT2 receptor is re-expressed in injured blood vessels and attenuates neointima formation [1]. Furthermore, a recent study clearly showed that selective stimulation of AT2 receptor induces vascular mass regression by stimulating VSMC apoptosis in spontaneously hypertensive rats in vivo [41]. Thus, SHP-1 might play an important role in AT2 receptor-mediated apoptosis,


