Angiotensin II modulates frizzled-2 receptor expression in rat vascular smooth muscle cells

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ABSTRACT

Ang II (angiotensin II) has multiple effects on vascular smooth muscle cells through the modulation of different classes of genes. Using the mRNA differential-display method to investigate gene expression in rat aortic smooth muscle cells in culture in response to 3 h of Ang II stimulation, we observed that Ang II down-regulated the expression of a member of the family of transmembrane receptors for Wnt proteins that was identified as Fzd2 [Fzd (frizzled)-2 receptor]. Fzds are a class of highly conserved genes playing a fundamental role in the developmental processes. In vitro, time course experiments demonstrated that Ang II induced a significant increase ($P < 0.05$) in Fzd2 expression after 30 min, whereas it caused a significant decrease ($P < 0.05$) in Fzd2 expression at 3 h. A similar rapid up-regulation after Ang II stimulation for 30 min was evident for TGFβ1 (transforming growth factor β1; $P < 0.05$). To investigate whether Ang II also modulated Fzd2 expression in vivo, exogenous Ang II was administered to Sprague–Dawley rats (200 ng · kg$^{-1}$ · min$^{-1}$; subcutaneously) for 1 and 4 weeks. Control rats received normal saline. After treatment, systolic blood pressure was significantly higher ($P < 0.01$), whereas plasma renin activity was suppressed ($P < 0.01$) in Ang II–treated compared with the saline-treated rats. Ang II administration for 1 week did not modify Fzd2 expression in aorta of Ang II–treated rats, whereas Ang II administration for 4 weeks increased Fzd2 mRNA expression ($P < 0.05$) in the tunica media of the aorta, resulting in a positive immunostaining for fibronectin at this time point. In conclusion, our data demonstrate that Ang II modulates Fzd2 expression in aortic smooth muscle cells both in vitro and in vivo.

INTRODUCTION

Ang II (angiotensin II) plays a key role in blood pressure regulation and cardiovascular homeostasis [1]. It has been demonstrated that Ang II is involved in a variety of non-haemodynamic processes acting on cell growth, atherothrombosis, apoptosis and inflammation [2]. Ang II, through an increase in blood pressure and its action as a true cytokine, contributes to the phenotypic changes that characterize vascular smooth muscle cells during the...
onset and progression of vascular damage in hypertension [3].

Overall, these effects of Ang II require a complex and dynamic pattern of modulation of gene expression. To investigate differential gene expression caused by Ang II in RASMC (rat aortic smooth muscle cells), we have used the mRNA differential-display method [4]. During the screening of genes in RASMC cultured without and with Ang II, we identified a cDNA sequence, down-regulated by 3 h of stimulation with Ang II, that showed a high similarity to the mouse Fzd (frizzled) genes. Fzds are a class of genes originally identified in developmental signalling pathways in *Drosophila*, and have been implicated in embryogenesis, tissue differentiation, cell proliferation and cell polarity [5,6]. Experimental data indicate that Fzds act as cell-surface receptors for Wnt proteins [7], a family of signalling molecules that regulate cell–cell interaction during embryogenesis and tissue differentiation. In addition, recent evidence indicates a role for Wnt/Fzd signalling in the formation and remodelling of the vasculature [8,9].

The aim of the present study was to establish the identity of our cDNA sequence, which was differentially modulated by Ang II in RASMC *in vitro*, and to investigate the effect of Ang II on Fzd2 (Fzd-2 receptor) expression in the tunica media of rat aortas *in vivo*.

**METHODS**

**Cell culture**

Primary RASMC isolated from 10–12-week-old male Sprague–Dawley rats were kindly provided by Professor A. Corsini (Department of Pharmacology, University of Milan, Milan, Italy). Cells (between passage five and 15) were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Celbio) supplemented with 10% fetal calf serum, glutamine (4 mmol/l), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere of 5% CO₂ in air until confluent, rinsed twice with SF (serum-free) medium; Celbio) diluted cDNA (100 ng) and amplified by PCR at 95 °C for 15 s and 60 °C for 1 min for 40 cycles.

**RNA extraction and reverse transcription**

Total RNA was extracted from cell culture and rat aortas using Trizol reagent (Invitrogen). A portion (2 µg) of RNA was reverse-transcribed to synthesize cDNA using SuperScript II Rnase H-Reverse Transcriptase (Invitrogen).

**mRNA differential display PCR**

Differential display comparing Ang II-stimulated cells and control cultures was performed as described previously [4]. The primer combination to amplify the cDNA subsequently identified as Fzd2 mRNA was T₁₁V₃ (where V is A or C or G) and CGACGGGGGCT. Because the originally amplified cDNA was 267 nt, RACE (rapid amplification of cDNA ends) [10] was performed, resulting in a cDNA sequence of 633 nt that was analysed by bioinformatics (www.ncbi.nlm.nih.gov/BLAST/; www.ncbi.nlm.nih.gov/mapview/) in order to evaluate homologies or similarity.

**Quantitative real-time PCR**

mRNA expression of Fzd2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), used as reference gene, was evaluated by quantitative multiplex real-time PCR using the TaqMan Realtime ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The primer sequences (Roche) used for rat Fzd2 amplification were: probe, FAM-CATCCTTCTGCTGGC-GGGCT-TAMRA (where Fam and TAMRA are 6-carboxyfluorescein and 6-carboxytetramethylrhodamine respectively); forward primer, CCGCTCTTCTGTG-ACCTGTTC; and reverse primer, CGGATGCGGAA-GAGTGACA (designed by Primer Express; Applied Biosystems).

A 25 µl reaction volume containing 900 nmol/l primers for Fzd2, 300 nmol/l primers for GAPDH, 200 nmol/l for each probe and 1 × Master Mix No AmpErase (Applied Biosystems) was mixed with 5 µl of diluted cDNA (100 ng) and amplified by PCR at 95 °C for 15 s and 60 °C for 1 min for 40 cycles.

**TGFβ1** (transforming growth factor β1) mRNA expression was evaluated using the Assay-on-Demand Gene Expression Product (Applied Biosystems) according to the manufacturer’s instructions.

The TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems) was used to measure GAPDH mRNA expression. The mRNA expression of target and reference genes was measured in triplicate for each sample. Values are reported as ratios of Fzd2/GAPDH mRNA and TGFβ1/GAPDH mRNA, and are expressed in arbitrary units.

**In vivo experiments**

Experiments were conducted in accordance with the Recommendation from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Experiments were performed in conscious 12-week-old male Sprague–Dawley rats (body weight, 200–250 g). Animals were housed individually in metabolic cages in a temperature-controlled room with a 12 h/12 h light/dark
cycle for the whole experimental period and were acclimatized with the metabolic cages and experimental procedures. Rats had free access to a standard rat chow and tap water. SBP (systolic blood pressure; mmHg) and body weight (g) were measured twice a week by an investigator who was blinded to the specific treatment. SBP was assessed by the tail cuff method (average of six recordings).

To evaluate the effect of Ang II administration for 1 week, 10 rats were implanted subcutaneously with osmotic minipumps (Alzet 2001; Alza) under sodium pentobarbital anaesthesia (40 mg/kg of body weight, intraperitoneally) in order to receive either Ang II at the dose of 200 ng·kg\(^{-1}\)·body weight·min\(^{-1}\) (Ang II treated; \(n = 6\)) or physiological saline (saline treated; \(n = 4\)) for 1 week. At the end of this period the rats were killed.

To evaluate the effect of Ang II administration for 4 weeks, a group of 13 rats (Ang II treated, \(n = 7\); saline treated, \(n = 6\)) were implanted subcutaneously with osmotic minipumps (Alzet 2004; Alza) to receive the same dose of Ang II described above or normal saline. At the end of the experimental period, all rats were decapitated and trunk blood was collected to measure PRA (plasma renin activity; ng angiotensin I·ml\(^{-1}\)·h\(^{-1}\)) by RIA. Aortas were immediately excised. For mRNA-expression studies, the media was separated from the adventitia and endothelium, snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). Aortic Fzd2 mRNA expression was evaluated by real-time PCR. An aortic ring was fixed with 10% formalin for histological examination and morphometric analysis, as described previously [11], and for immunohistochemical analysis. Aortic tissue, embedded in paraffin wax, was cut into 2 \(\mu\)m sections, dried at 65°C for 1 h, and then deparaffinized in xylene for 20 min, followed by dehydration through a series of graded alcohols. The endogenous peroxidase activity was blocked with 3% (v/v) \(\text{H}_{2}\text{O}_{2}\) in methanol for 20 min. Antigen was retrieved by heating the slides at 96°C in 10 mmol/l of sodium citrate buffer (pH 6.0) for 40 min. The aortic sections were then incubated for 60 min at room temperature with a polyclonal rabbit antibody against human fibronectin (dilution 1:50; Biomeda). Avidin–biotin–horseradish peroxidase was used to label the primary antibody. The reaction product was detected using 3,3′-diaminobenzidine. Control experiments were accomplished by omitting the primary antibody.

### Statistical analysis
Data are presented as means ± S.E.M. Data from the cell experiments were assessed with the use of two-way ANOVA with pairwise post-hoc comparisons using Fisher’s protected least-significant test. Differences between Ang II- and saline-treated rats for SBP, body weight, PRA, Fzd2 mRNA expression and collagen content in the tunica media were analysed by the unpaired Students’ \(t\) test. Differences between means were considered significant at \(P < 0.05\).

### RESULTS

#### Gene identification
Using differential-display analysis, we originally identified a 267 nt cDNA sequence down-regulated following stimulation by Ang II for 3 h in cultured RASMC. RACE experiments were performed resulting in a cDNA sequence of 633 nt (Figure 1A) that was analysed by bioinformatics in order to evaluate homology or similarity. The 633 nt sequence contained the high percentage (60.5%) of A/T typical of 3′-UTR (untranslated region) sequences.

A search in the rat genome database, using the BLAST program, indicated that the 633 nt sequence shared 98% identity with *Rattus norvegicus* chromosome 10 WGS supercontig (4731587 bp) from nt 383914–384518 (GenBank® accession no. NW_047340). As shown in Figure 2(A), our cDNA sequence was downstream of LOC360246, which indicates a hypothetical gene homologous with NM_172035 (GenBank® accession no.) that corresponds to rat Fzd2.

In addition, by searching GenBank® we found that our sequence shared 86–96% identity with MFzd2 mRNA (*Mus musculus* Fzd homolog 2; GenBank® accession no. BC049774) and 86–94% identity with MFzd10a mRNA (*M. musculus* Fzd10a; GenBank® accession no. AF206332). The 633 nt sequence identified matches with the 3′-UTR of both of these mouse Fzd genes (Figure 1B).

Since our cDNA sequence derived from cultured RASMC, we investigated whether there was any homology between mouse and rat sequences. We found that MFzd2 encodes for a protein sharing 97.89% identity with rat Fzd2 protein (NCBI accession no. Q08464), which is encoded by the mRNA of RATFRZH [R. norvegicus *Drosophila* polarity gene (*Fzd*) homologue; GenBank® accession no. L02530].

It should be considered that RATFRZH mRNA sequence is identical (100%) with rat Fzd2 mRNA sequence (GenBank® accession no. NM_172035), even if they have different accession numbers and different names in GenBank®. This is not surprising since, to date, the nomenclature of Fzd genes, as well as for Wnt genes, is not completely resolved (see Wnt gene homepage, http://www.stanford.edu/~rnusse/wntwindow.html).

Furthermore, as 1710 nt of the reported total 1912 nt sequence of RATFRZH/Fzd2 mRNA indicates the coding region, we hypothesized that our isolated 633 nt cDNA sequence might be the 3′-UTR of RATFRZH/Fzd2 mRNA.

When we performed an alignment using the BLAST program between the RATFRZH/Fzd2 sequence
Figure 1  Query cDNA sequence down-regulated in RASMC (A), and alignment of the sequence with Fzd genes (B)

(A) The cDNA sequence of the 633 nt down-regulated in RASMC identified by differential-display analysis. The polyadenylated signal sequence is underlined. (B) Alignment of Mfzd2 (i), Mfzd10a (ii) and the query cDNA sequence (iii). The alignment includes the 3′-UTR of the two mouse Fzd genes. Shaded regions indicate identical sequences between Mfzd2, Mfzd10a and the query sequence.

Since the 633 nt sequence also shares 86–94% identity with MFzd10a mRNA, we performed an alignment between this mouse gene sequence and the chromosome 10 WGS supercontig, and we observed a 85–94% identity

(GenBank® accession no. L02530 and NM_172035) and the R. norvegicus chromosome 10 WGS supercontig, we found that these two sequences shared 99% identity (Figure 2B).
in the same points in which we found an identity both with RATFRZH/Fzd2 mRNA and our 633 nt sequence (Figure 2B). We also found a similar identity (84–95 %) in the same position between MFzd2 mRNA and the WGS supercontig (Figure 2B).

This evidence, together with the localization on rat chromosome 10, supports further the hypothesis that our 633 nt sequence may be the 3'-UTR of rat RATFRZH/Fzd2 mRNA (Figure 2B).

**In vitro experiments**

Ang II induced a time-dependent modulation of Fzd2 mRNA expression in cultured RASMC (Figure 3). A significant increase ($P = 0.02$) in Fzd2 mRNA expression compared with cells receiving SF media alone was present at 30 min, whereas Ang II stimulation for 3 h caused a significant decrease ($P = 0.04$) in Fzd2 mRNA expression, confirming the data obtained by differential-display experiments. No differences in Fzd2 expression were observed at 1 h ($P = 0.37$) and 8 h ($P = 0.74$) when Ang II-treated cells were compared with cells receiving SF media alone.

In the same experiments, Ang II induced a similar rapid up-regulation of TGFβ1 mRNA expression at 30 min (0.245 ± 0.12 arbitrary units with SF alone compared with 1.748 ± 0.61 arbitrary units with Ang II; $n = 4$; $P = 0.02$). No differences were observed at 1 h (0.702 ± 0.59 arbitrary units with SF alone compared with 0.985 ± 0.42 arbitrary units with Ang II; $P = 0.66$) and 3 h (SF 1.333 ± 0.30 arbitrary units with SF alone compared with 0.693 ± 0.26 arbitrary units with Ang II; $P = 0.33$).
**In vivo effects of Ang II administration for 1 and 4 weeks**

After 1 week, SBP was significantly higher ($P < 0.01$) and PRA was significantly lower ($P < 0.01$) in Ang II-treated rats compared with the corresponding control animals (SBP, 163.6 ± 9.2 compared with 123.0 ± 5.2 mmHg respectively; PRA, 0.33 ± 0.3 compared with 2.88 ± 0.47 ng angiotensin I·ml⁻¹·h⁻¹ respectively). No differences in Fzd2 mRNA expression were present between saline- and Ang II-treated rats (1.23 ± 0.11 compared with 1.21 ± 0.24 arbitrary units respectively; $P = 0.92$).

At the end of the treatment period, body weight in the saline-treated group was higher than in Ang II-treated group (311.0 ± 3.10 compared with 286.5 ± 5.65 g respectively; $P = 0.01$). Ang II treatment for 1 week did not cause any histological modification of the vascular wall (Figure 4A) or any differences in collagen content (12.8 ± 1.8% fibrosis in the saline-treated rats compared with 15.0 ± 1.9% fibrosis in the Ang II-treated rats; $P = 0.45$; Figure 4B).

After 4 weeks of treatment with Ang II, SBP was significantly higher ($P < 0.01$), whereas PRA was markedly lower ($P < 0.01$), in Ang II- compared with saline-treated rats (SBP, 218.4 ± 4.8 compared with 136.7 ± 10.4 mmHg respectively; PRA, 0.03 ± 0.01 compared with 2.53 ± 0.57 ng angiotensin I·ml⁻¹·h⁻¹ respectively). Fzd2 mRNA expression was significantly higher in the tunica media of rat aortas in Ang II- compared with saline-treated rats (0.59 ± 0.08 compared with 0.32 ± 0.06 arbitrary units respectively; $P < 0.05$). At the end of the treatment period, body weight in the saline- and Ang II-treated groups were similar (407.7 ± 9.5 compared with 383.1 ± 14.6 g respectively; $P = 0.20$). Ang II treatment for 4 weeks did not cause any histological modification in the vascular wall (Figure 5A), or any differences in collagen content (18.0 ± 2.0% fibrosis in saline-treated animals compared with 15.0 ± 1.0% fibrosis in Ang II-treated animals; $P = 0.16$; Figure 5B). Ang II treatment for 4 weeks caused an increase in fibronectin expression in rat aortas (Figure 5C).

**DISCUSSION**

A link between Ang II and the Fzd gene family in vascular smooth muscle cells was identified in our experiments by differential-display mRNA expression analysis. Fzd, which have a seven-transmembrane structure similar to AT2 (Ang II type 2) receptors [12], are widely expressed in the cardiovascular system. They act as receptors for Wnt proteins, which play a major role in the developmental processes in different organs [13,14]. Members of Wnt family are multifunctional secreted glycoproteins involved in cell differentiation, proliferation, migration, cell polarity and apoptosis [15,16]. Wnt/Fzd binding, when involving Fzd1, activates the cytoplasmic protein dishevelled and modulates the ability of β-catenin to activate the transcription of specific target genes (an up-to-date list of target genes is reported on the Wnt Gene homepage at http://www.stanford.edu/~rnusse/wntwindow.html). In contrast,
when Wnt/Fzd binding involves Fzd2, stimulation of calcium transients and the activation of PKC (protein kinase C) and calcium/calmodulin kinase II in a β-catenin-independent manner occurs. Wnt/Fzd interaction is controlled by a family of extracellular secreted proteins, the Fzd-related proteins, which act as antagonists of Wnt signalling [17].

To date, as the gene sequence reported in GenBank® for rat Fzd2 (the same as for RATFRZH) corresponds to the coding region (1710 nt out of a total of 1912 nt reported), we cannot directly find our 633 nt (3′-UTR) in this sequence. However, our search to find the homologies of our 633 nt sequence strongly indicates that it might identify the 3′-UTR region of the rat Fzd2 gene. The position of our 633 nt on rat chromosome 10, downstream of LOC360246, and the high homology but not the identity, because we compared rat and mouse genes, between the 3′-UTR region in our 633 nt sequence and the MFzd2 and MFzd10a support our conclusion.

It has been demonstrated that cultured RASMC express Fzd2 mRNA [18]. In our experimental conditions, Ang II, a potent growth factor, showed a time-dependent modulation of Fzd2 mRNA expression in cultured RASMC. Ang II caused a significant increase in Fzd2 expression after 30 min of stimulation, whereas it caused a significant decrease in Fzd2 expression at 3 h. A similar rapid increase at 30 min after Ang II stimulation is evident for TGFβ1, which is known to influence the growth of RASMC. This co-ordinated expression induced by Ang II between Fzd2 and TGFβ1 suggests the existence of an interaction between these genes, indicating the existence of a crosstalk between TGFβ and Wnt pathways, as has been described in developmental processes [19]. In vivo, Ang II administration for 1 week did not modify Fzd2 expression, whereas Ang II administration for 4 weeks caused an up-regulation in Fzd2 mRNA expression in RASMC.

Our present findings are consistent with other experimental data demonstrating that, in quiescent cultured RASMC, stimulation with growth factors, such as PDGF-BB (platelet-derived growth factor-BB) and FGF-2 (fibroblast growth factor-2), caused a down-regulation of Fzd2 mRNA expression at 4 h of treatment [18].

Interestingly, the same authors [18] demonstrated that Fzd2 mRNA expression in vivo showed a transient down-regulation in rat aorta in the first few hours after balloon injury, whereas 3 weeks after balloon injury Fzd2 mRNA expression was increased, suggesting that Fzd2 might have a role in smooth muscle cell alignment during vascular remodelling processes. In our in vivo experimental model, Fzd2 in the aortic media showed a similar expression pattern. In fact, although Ang II administration for 1 week did not modify Fzd2 mRNA expression, Ang II administration for 4 weeks induced an up-regulation of Fzd2 mRNA. In our experimental conditions, we did not find any histological modification in the aortas of Ang II-treated rats. A possible explanation for this might be that large arteries, such as aorta, slowly develop structural modifications in response to Ang II, whereas small vessels, i.e. mesenteric arteries, rapidly develop histological modifications in response to Ang II administration [20]. However, in aortas of rats treated with Ang II for 4 weeks, we observed an increase in fibronectin expression, an extracellular matrix protein implicated in vascular remodelling processes, indicating an early shift in the phenotype of vascular smooth muscle cells [21,22].

In our experimental model, a possible role of increased blood pressure caused by Ang II on Fzd2 mRNA expression cannot be excluded, even if modulation of Fzd2 expression was demonstrated in vitro at first, where
haemodynamic effects are absent, and to date no evidence has been provided for a role of blood pressure on Fzd2 expression in vascular tissue.

In agreement with our present results, other experimental data have demonstrated an up-regulation of Fzd-2 mRNA expression in remodelling processes in cardiomyocytes during the development of left ventricular hypertrophy [23], in mouse myocardial tissue during ischaemia [24] and in myofibroblasts during their migration in rat infarcted heart [25], suggesting a potential involvement of Wnt/Fzd pathway in the cardiovascular remodelling processes.

In conclusion, our present data have shown a novel unexpected link between Ang II and Fzd genes and strongly indicate that the Wnt/Fzd system might be a further intracellular signalling pathway for Ang II in vascular smooth muscle cells, with possible implications for Ang II-dependent vascular remodelling.

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