

Activation of the cell membrane angiotensin AT₂ receptors in human leiomyosarcoma cells induces differentiation and apoptosis by a PPAR γ – dependent mechanism

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Received July 13, 2016 / Accepted November 25, 2016

Angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system (RAS), acting on AT₁ and AT₂ receptors participates in the regulation of proliferation, differentiation and apoptosis in tumour cells. The peroxisome-proliferator activated receptor γ (PPAR γ) and its ligands exert anti-tumour effects in various human cancer cell lines. The present study investigates the effects initiated by AT₁- and AT₂ receptor stimulation in SK-UT-1 cells, a human leiomyosarcoma cell line, and clarifies the role of the PPAR γ in the AT₂ receptor-induced differentiation and apoptosis.

Selective stimulation of AT₁- and AT₂ receptors was achieved by incubation of the cells with Ang II (10⁻⁶ M) in the presence of the selective AT₂ receptor antagonist, PD 123177 (10⁻⁶ M) and the AT₁ receptor antagonist, losartan (10⁻⁵ M), respectively, the selective PPAR γ antagonist, GW 9662, was used at concentration 10⁻⁶ M. The expression of smooth muscle cell differentiation markers, SM22 α and calponin, was analysed at RNA- and protein levels using RT PCR and Western blot, which was also used to quantify Bcl-2-, Bax- and cleaved caspase-3 proteins. The translocation of the AT₂-receptor interacting protein 1 (ATIP1) to the nuclei was studied by Western blot and immunofluorescence staining. The mitochondrial status and the metabolic activity in response to AT₁- and AT₂ receptor activation were assessed by the quantification of ^{99m}Tc – sestamibi and 2'-deoxy-2'-[¹⁸F]fluoro-D-glucose uptake.

AT₁ receptor stimulation did not exert any profound effects in quiescent SK-UT-1 cells. The effects induced by Ang II acting on AT₂ receptors were time-dependent. A short, 3 – 6 h lasting stimulation promotes differentiation, i.e. increases in the mRNA- and protein levels of SM22 α and calponin, whereas a sustained stimulation for 48 h activates the intrinsic apoptotic pathway, as evidenced by reduced cell numbers, down-regulation of the anti-apoptotic Bcl-2 protein and increased levels of the Bax protein and cleaved caspase-3. The effects were reversed by the PPAR γ antagonist, GW 9662, clearly implying a PPAR γ -dependent mechanism. Our results also demonstrate a co-localisation of the AT₂-receptor interacting protein, ATIP1, and the PPAR γ in nuclei of SK-UT-1 cells and an accumulation of ATIP1 in the nuclear fraction in response to AT₂ receptor stimulation. The regulation of the differentiation and apoptosis via the AT₂ receptor favours an important functional role of this receptor in quiescent, slow-cycling SK-UT-1 cells and provides the rationale for the use of AT₁ receptor antagonists for the treatment of human leiomyosarcomas.

Key words: Angiotensin AT₂ receptor, uterine leiomyosarcoma, differentiation, apoptosis, PPAR γ , ATIP1

Local tissue renin-angiotensin systems (RAS), which have been identified in most organs such as in the kidney, brain, liver and female reproductive organs, participate in the regulation of cellular proliferation and apoptosis [1]. The RAS components were also detected in human cancers [2]. Angiotensin II (Ang II), the main effector peptide of RAS, exerts its effects via bind-

ing to at least two seven-transmembrane domain receptors, angiotensin type 1 (AT₁) and type 2 (AT₂) receptor. The AT₁ receptor in malignant tumours is often up-regulated during the progression from normal to malignant phenotypes and its over-expression correlates with tumour growth and metastasis. Ang II acting on the AT₁ receptor is a potent growth – pro-

moting factor, enhances proliferation and invasion of cancer cells and promotes tumour angiogenesis [2, 3]. Generally, the AT₂ receptor mediates effects opposing and counterbalancing those initiated by the AT₁ receptors, e.g. it inhibits cellular proliferation and migration and counteracts growth-promoting actions of growth factors and the AT₁ receptor [3, 4]. AT₂ receptor activation or its over-expression attenuated cell growth and promoted apoptosis in lung adenocarcinoma cells or in rat pheochromocytoma (PC12W) cells [5, 6]. In contrast to the AT₁ receptor, the AT₂ receptor does not couple to the heterotrimeric G-proteins, but interacts with different AT₂-receptor interacting proteins (ATIPs) which mediate the biological effects of AT₂ receptor activation [7, 8]. ATIPs are encoded by a single gene MTUS1 (microtubule associated tumor suppressor 1) and five MTUS1 transcript variants have been classified in three groups, ATIP1, ATIP3 and ATIP4. All ATIPs isoforms share a common AT₂ receptor interacting domain and act, especially the ATIP1, as mediators of the growth-inhibitory actions initiated by the AT₂ receptor [9]. For instance, ATIPs expressed in human prostate cancer cell lines were demonstrated to mediate the AT₂ receptor-induced anti-growth effects [10, 11].

The peroxisome proliferator-activated receptor γ (PPAR γ), a ligand activated transcription factor, is an important regulator of glucose and lipid metabolism. The synthetic PPAR γ activators thiazolidinediones, such as pioglitazone, decrease insulin resistance in the adipose tissue, skeletal muscles and the liver [12]. The PPAR γ is also expressed in various human tumours, including lung, breast and colon cancers and gynaecological malignancies. Activation of PPAR γ inhibits cellular proliferation and induces differentiation and/or apoptosis indicating a role for the receptor as a potential tumour suppressor [13, 14]. Experimental data indicates a link between the RAS and the PPAR γ in vascular smooth muscle cells (SMC) or tumour PC12W cells [15-17]. Thus, the interplay between angiotensin receptors and the PPAR γ may considerably contribute to the modulation of processes associated with proliferation, differentiation or apoptosis of cancer cells.

We have recently reported that AT₂ receptors in SK-UT-1 cells, a human leiomyosarcoma cell line, are up-regulated in quiescent state [18]. The present study conducted in quiescent, slow-cycling SK-UT-1 cells investigates the effects of AT₁- and AT₂ receptor stimulation with Ang II on proliferation, differentiation and/or apoptosis and clarifies the role of the PPAR γ in these processes. Ang II binds with high affinity to the AT₁- and the AT₂ receptor (IC₅₀ = 0.2 nM and 0.6 nM, respectively). To selectively activate the membrane AT₁- and AT₂ receptors, SK-UT-1 cells were exposed to Ang II and the high-affinity AT₂ receptor antagonist, PD 123177 (IC₅₀ = 34 nM) and the AT₁ receptor antagonist, losartan (IC₅₀ = 19 nM), respectively. These experimental conditions ensure an effective and selective activation of both subtypes of the membrane angiotensin receptors. The uptake of ^{99m}Tc – sestamibi (^{99m}Tc-MIBI) and 2'-deoxy-2'-[¹⁸F] fluoro-D-glucose (¹⁸F-FDG) were used to assess the mitochondrial status and the metabolic

activity after stimulation of AT₁- and AT₂ receptors [19, 20]. To explore the role of the PPAR γ in the mediating of effects initiated by the AT₂ receptor stimulation, we employed GW 9662, a potent, high-affinity PPAR γ antagonist. Our results demonstrate that AT₂ receptor stimulation in SK-UT-1 cells induces differentiation and activates mitochondrial apoptotic pathway by a PPAR γ – dependent mechanism and suggest a role of ATIP1 in mediating these effects.

Materials and methods

Chemicals. Angiotensin receptor ligands: Ang II was purchased from Sigma-Aldrich (Taufkirchen, Germany). Losartan was generously supplied by Dr. R. Smith (DuPont Merck Pharmaceutical Company, Wilmington, DE, USA). PD 123177 was a kind gift from Joan Keiser (Park Davis, Ann Arbor, Michigan, USA). Ang II, losartan and PD 123177 were dissolved in ultra-pure water. GW 9662, a potent and selective PPAR γ antagonist, purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA) was dissolved in DMSO and ultra-pure water. All other chemicals, antibodies and kits are mentioned in the text and if not otherwise stated, they were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Cell culture. The human leiomyosarcoma cell line, SK-UT-1 cells (HTB-114, American Culture collection) was obtained from the European Collection of Animal Cell Cultures. SK-UT-1 cells have been established and characterised as a cell line, which can grow in vitro and produce tumours in nude mice after transplantation. This cell line is most frequently used to study the biology of the human leiomyosarcoma and to test the effects of various anticancer drugs. The cells, kindly provided by Dr. Hendrik Ungefroren, University of Hospital of Schleswig-Holstein, Campus Kiel, Germany [21], were cultured in a culture medium consisting from DMEM and HAM' -12, 1:1 (Invitrogen GmbH, Darmstadt, Germany) supplemented with 10 % heat inactivated foetal calf serum (FCS, Biowhittaker Bioproducts Walkersville MD, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM of L-glutamine. All cells grew as a monolayer in dishes at 37 °C in a humidified atmosphere of air/CO₂ (19:1). The cell line was passed using 1.5 ' trypsin/EDTA (0.05% trypsin and 0.02% EDTA). To obtain quiescent cells, SK-UT-1 cells were maintained in a medium containing 0.1% FBS for 48 hours before the experiments.

RNA isolation and RT-PCR. Total RNAs were extracted with Trizol-reagent and dissolved in RNase-free water. The RNA quantity and quality were measured using a spectrophotometer. First-strand synthesis (5 μ g total RNA) was carried out with the Superscript Reverse Transcription Kit using Oligo-dT₁₂₋₁₈ oligonucleotides (Invitrogen GmbH, Darmstadt, Germany). The equality of the reverse-transcribed cDNA was verified by RT-PCR using intron spanning primers for β -actin (Clontech, Saint-Germain-en-Laye, France). All PCR reactions were carried out under standard conditions

unless otherwise stated. The PCR temperature profile used was 94 °C (5 minutes) hot start, followed by 30 cycles at 94 °C (45 s), 56-60°C (45 s), 72 °C (1 min), and 7 minutes at 72 °C. Optimal PCR conditions were established by determining of the ratio between the signal strength and the number of PCR cycles. The saturation of PCR reactions were reached at 33 PCR cycles for β -actin, at 25 cycles for SM22 α and calponin (data not shown). The PCR products were separated by electrophoresis on 1.0-1.5% agarose gels, stained with 0.5 mg/ml ethidium bromide and visualized by UV illumination. The amplification for the β -actin control was verified for the correct size (1128 bp) to ensure the quality of cDNA and lack of contamination with genomic DNA. Autoradiographic signals were densitometrically quantified (Bio-Rad Image software, Quantity One, Bio-Rad Laboratories; Hercules, CA).

Oligonucleotides. The following oligonucleotides were used for RT-PCR cDNA: β -actin: sense (5'-ATG GAT GAT GAT ATC GCC GCG-3') and antisense primers (5'-CAT GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3'); calponin: sense (5'-AAA CAG GTG AAC GTG GGA G-3') and antisense primers (5'-TAG TTG TGT GCG TGG TGG TT-3') were used for PCR amplification of to yield a 528-bp product; SM22 α : sense (5'-ACA AGT CTT CAC TCC TCC CTG C-3') and antisense primers (5'-TCA AAG AGG TCA ACA GTC TGG A-3') were used for PCR amplification of to yield 408-bp product.

Western blot analysis. Cultured SK-UT-1 cells were lysed in CellyticTM MT cell lysis reagent (Sigma Deisenhofen, Germany) containing 1% of the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific Rockford, IL, USA). After short incubation (5 min at 95° C), the lysates were briefly sonicated and centrifuged (15,000 x g at 4 °C for 15 min) to remove insoluble materials. The protein concentration in the supernatant was measured by BCATM protein assay kit (Thermo Scientific, Rockford, IL, USA). Extracts equivalent to 20 μ g of total proteins per lane were loaded and separated on 12% SDS-polyacrylamide gels and transferred to Immobilon-P polyvinylidene difluoride membrane (PVDF) (Millipore; Billerica, MA, USA). The membranes were blocked and incubated overnight with the primary antibodies: monoclonal antibody against calponin (1:2000; Sigma, Deisenhofen, Germany), polyclonal antibody against SM22 α (1:10,000; Abcam, Cambridge, UK), monoclonal antibody against cleaved (activated) caspase 3 (1:1000; Cell Signal Technology, Danvers, MA, USA), monoclonal anti-Bcl-2 antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal anti-Bax antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the following day, the membranes were washed and incubated with the horseradish peroxidase-conjugated secondary antibody. Western blots were developed with ECL western blot detection reagents on high performance chemiluminescence film (Amersham International plc, Amersham; Piscataway, NJ). For the re-staining, the blots were stripped in the Restore Western Bolt Stripping Buffer (Thermo Scientific, Rockford,

IL, USA) and washed in PBST. To normalise the protein content of each lane, all membranes were stained with protein staining kit or blotted with anti- β -actin antibody (1:10,000; Sigma, Deisenhofen, Germany). The films were scanned and quantified using the quantification software (Quantity One, Bio-Rad Laboratories; Hercules, CA).

Cell counting. SK-UT-1 cells cultured in 24 well plates were washed with PBS and detached from the culture dish with trypsin/EDTA solution. The cell suspension was centrifuged at 110 g for 5 min, the pellets were re-suspended in PBS and mixed 1:1 with Trypan Blue solution 0.4% (w/v). The cell counting was carried with a haemocytometer.

Lactate dehydrogenase (LDH) assay. The assessment of cell membrane integrity and cell death based on the measurement of LDH activity released from the cytosol of damaged cell into the culture medium was determined by the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany).

Preparation of the nuclear fraction. Cultured SK-UT-1 cells were washed with PBS, detached from the culture dish with trypsin/EDTA solution and centrifuged at 500 x g for 5 min. The pellets were re-suspended with PBS, centrifuged again at 500 x g and the supernatant was discarded. The nuclear protein extracts were obtained using "NE-PER Nuclear and Cytoplasmic Extraction Reagents" (Thermo Scientific, Rockford, USA) according to the manufacturer's instruction. The protein extracts were stored at -80 °C.

Uptake of ^{99m}Tc - sestamibi (^{99m}Tc-MIBI). ^{99m}Tc-MIBI was synthesised using the Technescan Sestamibi 1 mg-Kit (Mallinckrodt, Dublin, Ireland) according to manufacturer's instruction and diluted with culture medium to 10 MBq/ml. After treatment of SK-UT-1 cells with angiotensin receptor ligands with or without GW 9662 for 48 h, 4 - 5 μ l of ^{99m}Tc-MIBI (80-100 KBq) were added to each well and the cells were incubated for 10 min. Then, the cells were rinsed 4-times with ice-cold PBS, detached from the culture dishes with 200 μ l of 0.05% trypsin/EDTA and shaken in a shaker for 3 min. Afterwards 200 μ l of lysis buffer containing protease inhibitor cocktail were added to each well. The cell lysates were then transferred to the 3 ml-silicon tube and the radioactivity was measured using a γ -counter (PerkinElmer). The lysates were then stored at 20 °C for protein determination. Non-specific binding was determined in the lysis buffer without cells. Radioactivity was corrected for the decay and the percentage of the radioactivity associated to the cells was calculated as follows: the percentage of ^{99m}Tc-MIBI uptake = CPM of cell lysate x 100/ the total radioactivity (CPM) added to the well. The counts in individual samples were normalised for protein content.

Uptake of 2'-deoxy-2'-[¹⁸F]fluoro-D-glucose (¹⁸F-FDG). Following stimulation of AT₁ or AT₂ receptors for 24 h or 48h, the cells were incubated in glucose - free medium for 4 h. Then, ¹⁸F-FDG (80-100 Kbbq/well, Eckert & Ziegler, Berlin, Germany) was added into the wells. After incubation for 20 min at 37 °C, the cells were rinsed 4-times with ice-cold PBS to remove the excess of the tracer and lysed with 200 μ l of 0.1 N NaOH in a shaker for 20 min. The radioactivity in the cell

lysates was measured using a γ -counter. The cell lysates were then neutralized with an equal amount of 0.1 N HCl, stored at -20 °C and used for protein measurements. The counts of the samples were corrected for the protein content.

Immunofluorescence staining for the PPAR γ and ATIP1.

Quiescent SK-UT-1 cells grown on cover slips were fixed with 4 % paraformaldehyde for 30 min at room temperature (RT), washed 3 times with PBST and permeabilised with 0.1 % Triton X-100 in 0.1 % sodium citrate for 5 min at RT. After incubation in the block solution containing 1% BSA, the cells were incubated with the first primary antibody mouse anti-human PPAR γ receptor antibody (1:400) at 4°C overnight. Following a wash step with PBST, the cells were washed with PBST and incubated with the second primary antibody rabbit anti-human ATIP1 (1:200) at 4 °C overnight. The cells were then washed with PBST and incubated with the secondary antibodies, Alexa Fluor® 488 -conjugated donkey anti-rabbit antibody and Alexa Fluor® 546- conjugated donkey anti-mouse antibody (Molecular Probes, Eugene, Oregon, USA) in 1% BSA. The cells were then washed 2-times with PBST, once with PBS, dried and mounted in ProLong® Gold Antifade Mountant with DAPI (4',6-diamidino-2'-phenylindole dihydrochloride) (ThermoFisher Scientific, Rockford, IL, USA). Immunofluorescence analyses were carried out by a fluorescence microscope (Leica DMR, Germany).

Protocols. Selective stimulation of angiotensin AT $_1$ and AT $_2$ receptors was achieved by a concomitant treatment of SK-UT-1 cells with Ang II (10⁻⁶ M) and the high-affinity, AT $_2$ - or AT $_1$ receptor antagonist, PD 123177 (10⁻⁶ M) and losartan (10⁻⁵ M), respectively. The PPAR γ antagonist, GW 9662, was used at a concentration of 10⁻⁶ M. The antagonists were added to cells 30 min before the application of the agonists. The concentrations of Ang II and the antagonists were established in preliminary experiments and employed in previous studies [18].

Differentiation study. The detection of SM22 α and calponin mRNAs was carried out in quiescent SK-UT-1 exposed to vehicle, Ang II \pm losartan or PD 123317 for 1, 3 and 6 h; proteins for Western blot analysis of SM22 α (n = 5) and calponin (n = 6) were isolated after AT $_1$ - and AT $_2$ receptor stimulation for 3, 6, 9 12 and 24 h. In the second set of experiments, quiescent SK-UT-1 cells were treated with vehicle or Ang II + losartan with or without the PPAR γ antagonist, GW 9662, for 9 h. Total proteins were isolated and used for Western blot analysis of calponin (n = 10/group) and SM22 α (n = 11/group).

Effects of a long-term activation of angiotensin receptors in SK-UT-1 cells. The effects of AT $_1$ - and AT $_2$ receptor stimulation on cell numbers and the ¹⁸F-FDG uptake were assessed in SK-UT-1 cells exposed to AT $_1$ - and AT $_2$ receptor ligands for 24 h (cell counting: n = 8/group; ¹⁸F-FDG uptake: n = 16/group) or 48 h (cell counting: n = 8/group; ¹⁸F-FDG uptake: n = 9/group).

Apoptosis. The highest apoptotic rates occur between 36 and 48 h after the onset of AT $_2$ receptor activation [18]. Apop-

totic processes in the present study were, therefore evaluated at 48 h after stimulation of the membrane angiotensin receptors. LDH release into the incubation media was determined in SK-UT-1 cells after AT $_1$ - and AT $_2$ - receptor stimulation for 48 h (n = 9/group) . Total proteins were isolated and used for quantification of the Bax- (n = 6) and Bcl-2 (n = 6) proteins and the activated caspase-3 (n = 5). To investigate the potential role of the PPAR γ in the AT $_2$ receptor - induced apoptosis, the cell numbers (n = 11/group) and the uptake of ^{99m}Tc-MIBI (n = 11/group) were assessed in quiescent SK-UT-1 cells incubated with vehicle and Ang II + losartan with or without GW 9662 for 48 h (cell counting: n = 11/group; ^{99m}Tc-MIBI: n = 11/group). The ongoing apoptosis was documented by quantification of Bax- and Bcl-2 proteins and activated caspase-3 (n = 6/group for each protein).

PPAR γ – ATIP1 interaction. The intracellular localisation of the PPAR γ and ATIP1 was studied by immunofluorescence staining in SK-UT-1 cells treated with vehicle (controls) or after AT $_2$ receptor stimulation.

ATIP1 was quantified by Western blot in the nuclear protein fraction isolated from SK-UT-1 cells exposed to *vehicle* or Ang II + losartan (selective activation of AT $_2$ receptors) for 3, 6, 12 and 24 h (n = 5 – 6 for each time point).

Statistical analysis. All values are expressed as the means \pm SEM. The numbers of separate experiments are given in brackets. The distribution of the sampled data, except the data of the ATIP1 protein values in the nuclear fraction, was analysed by Kolmogorov-Smirnov test. The statistical evaluation of the data was carried out by one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test for pairwise comparisons. Due to small sample size, not normally distributed data (Shapiro-Wilks-test) and apparent differences in variances between the values of two time courses of the ATIP1 protein in the nuclear fraction, nonparametric statistical tests were used. The effects of time on the ATIP1 protein values (longitudinal comparisons) were evaluated by Friedman test. Multiple pairwise semiparametric comparisons to the control group (0 h) were performed by Holm-Bonferroni method. The differences between the time courses (vehicle group vs. AT $_2$ receptor-stimulated group) were tested at each time point (3, 6, 12 and 24 h) by Wilcoxon-Mann-Whitney-Test. P values were adjusted for multiple testing by the factor 4.

Results

Stimulation of AT $_2$ receptors in quiescent SK-UT-1 cells induces differentiation at early time points by a PPAR γ – dependent mechanism. Stimulation of AT $_2$ receptors in SK-UT-1 cells up-regulated the mRNA levels of SM22 α and calponin, activation of AT $_1$ receptors was without effect (Figure 1A). An increase in the SM22 α and calponin mRNA levels was detected at 1 h, but not at 3 or 6 h after the treatment had started. Activation of AT $_2$ receptors significantly augmented the SM22 α and calponin proteins only at one time point, 9 h (SM22 α : F_{3,16} = 4.419, P<0.05; calponin: F_{3,20} = 4.130; P<0.05)

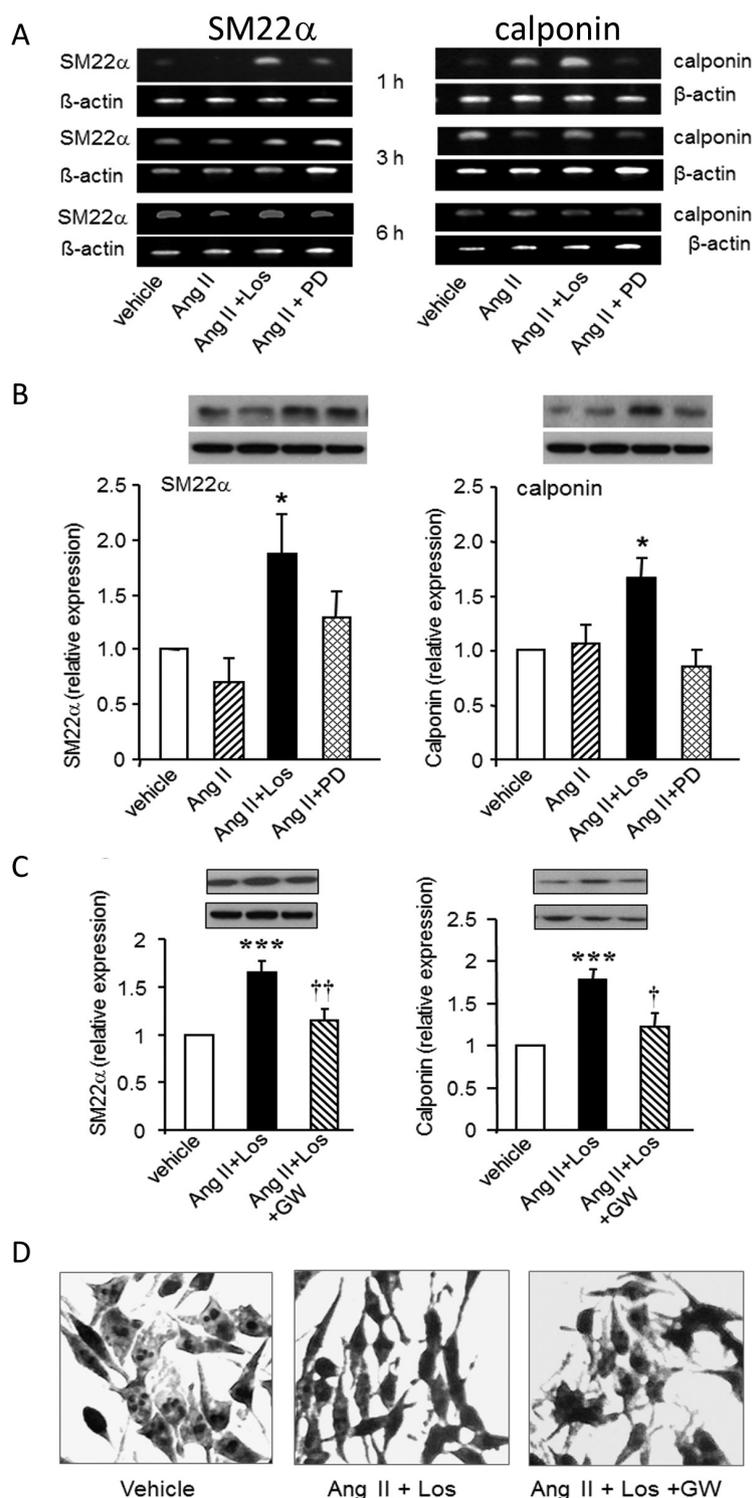


Figure 1. Ang II induces differentiation in SK-UT-1 cells via AT₂ receptor activation. (A) upper panels: RT-PCR to determine the time course of SM22 α - (left) and calponin (right) mRNA expression after AT₁- and AT₂ receptor stimulation. Panels (B) show the representative blots and the histograms of Western blot analysis of the SM22 α - and calponin proteins after AT₁- and AT₂ receptor stimulation for 9 h. Activation of AT₂ receptors significantly up-regulated SM22 α and calponin in SK-UT-1 cells. Statistical comparison with vehicle treated cells: * $P < 0.05$. (C) panels: Effects of the PPAR γ antagonist, GW 9662 (GW), on the AT₂ receptor-induced up-regulation of SM22 α and calponin. Depicted are the representative blots and the histograms. ** $P < 0.01$, *** $P < 0.001$, statistical comparison with vehicle treated cells, and † $P < 0.05$, †† $P < 0.01$, with cells treated with Ang II + losartan (Los), calculated by one-way ANOVA followed by a post hoc Bonferroni test for pairwise comparisons. Results are expressed as the means \pm SEM. Ang II: angiotensin II, Los: losartan, PD: PD 123177. (D) Structural features detected in quiescent SK-UT-1 cells after vehicle treatment (controls), AT₂ receptor activation or AT₂ receptor activation and concomitant inhibition of the PPAR γ .

(Figure 1B). We did not observe any changes in SM22 α and calponin protein levels either at earlier (3 and 6 h) or later time points (12 and 24 h) (data not shown). Selective inhibition of the PPAR γ by GW 9662 prevented the AT $_2$ receptor - induced up-regulation of SM22 α ($F_{2,27} = 8.798$, $P < 0.01$) and calponin ($F_{2,30} = 11.354$, $P < 0.001$) (Figure 1C).

The morphological changes related to the differentiation of SK-UT-1 cells upon AT $_2$ receptor stimulation were also observed (Figure 1D). Generally, SK-UT-1 cells are highly malignant cells and the rate of cell division in starvation media is high. Cells exposed to vehicle show disorganised arrangement, variation in size and shape, loss of normal features and mitoses. Activation of AT $_2$ receptors promotes differentiation as evidenced by the typical spindle form and anchorage dependence. The co-treatment with the PPAR γ inhibitor, GW 9662, almost completely reversed the effects induced by AT $_2$ receptor stimulation (Figure 1D).

Effects of a sustained AT $_1$ - and AT $_2$ receptor stimulation for 24 and 48 h on cell numbers and ^{18}F -FDG uptake into SK-UT-1 cells. In line with previous findings, stimulation of AT $_1$ - or AT $_2$ receptors for 24 h did not alter the cell numbers (Figure 2, left upper panel) [18]. Activation of AT $_1$ receptors for 24 h accelerated the ^{18}F -FDG incorporation by SK-UT-1 ($F_{3,60} = 3.458$, $P < 0.05$) cells indicating higher mitotic activities (Figure 2, left lower panel), stimulation of AT $_2$ receptors did not exert any effect. Ang II acting on AT $_2$ receptors for 48 h

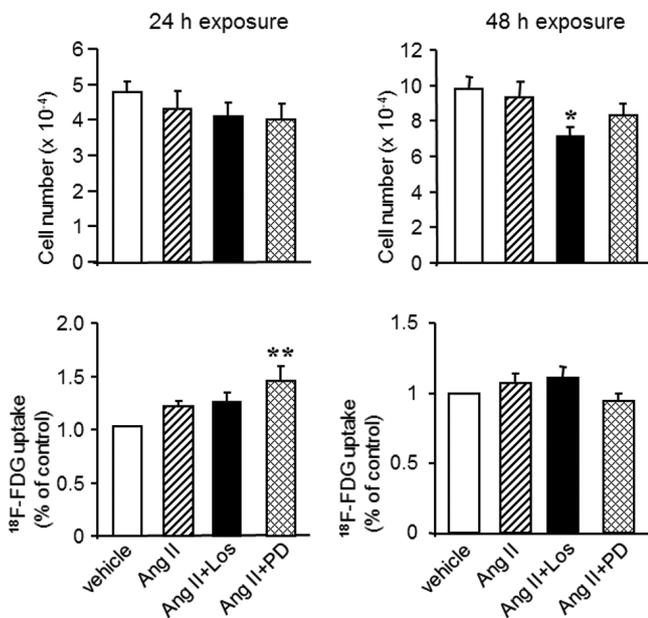


Figure 2. Effects of Ang II receptor activation on the cell numbers and ^{18}F -FDG uptake. Cells were treated with vehicle (control group), Ang II, Ang II + losartan (Los; stimulation of AT $_2$ receptors) and Ang II + PD 123177 (PD, selective stimulation of AT $_1$ receptors) for 24 h (left panels) or 48 h (right panels). * $P < 0.05$, ** $P < 0.01$, statistical comparison with vehicle-treated cells, calculated by one-way ANOVA followed by a post hoc Bonferroni test for pairwise comparisons. Results are expressed as the means \pm SEM.

reduced the numbers of SK-UT-1 cells ($F_{3,28} = 2.786$, $P < 0.05$) (Figure 2, right upper panel). A sustained stimulation of both Ang II receptor subtypes for 48 h did not modify the uptake of ^{18}F -FDG into the quiescent SKJ-UT-1 cells ($F_{3,32} = 1.463$, $P = 0.243$, Figure 2, right lower panel).

Ang II acting on the membrane AT $_2$ receptors induces apoptosis in SK-UT-1 cells by a PPAR γ -dependent mechanism. Prolonged incubation (≥ 48 h) in serum-deprived medium led to spontaneous apoptotic cell death, as evidenced by the detectable amounts of activated caspase-3 in vehicle-treated SK-UT-1 cells. Bcl-2 and Bax proteins play a crucial role in molecular pathways linked to the mitochondria - mediated apoptosis. Exposure of SK-UT-1 cells Ang II for 48 h aug-

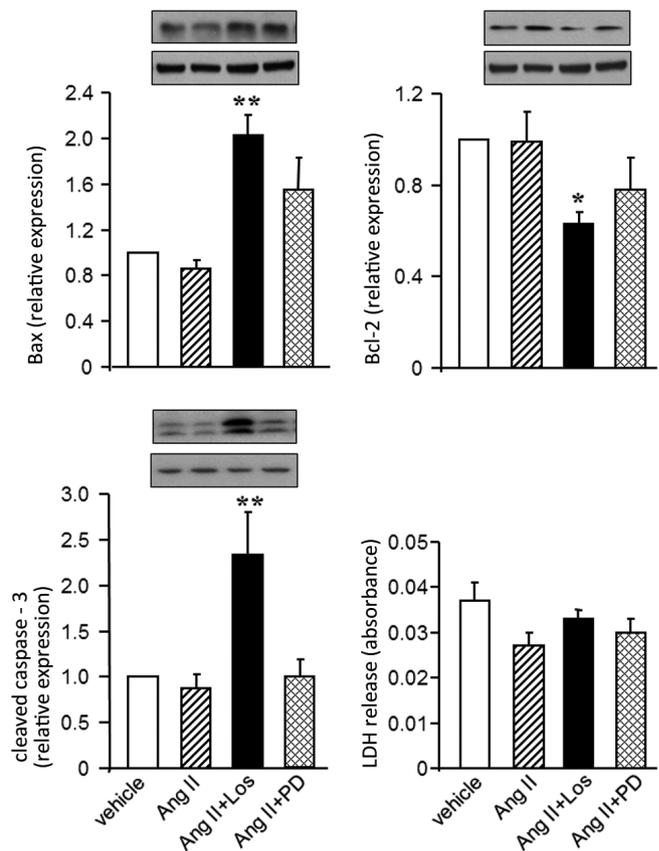


Figure 3. Effects of Ang II receptor activation upon apoptosis induction in SK-UT-1 cells. Cells were treated with vehicle (control group), Ang II, Ang II + losartan (Los, stimulation of AT $_2$ receptors) and Ang II + PD 123177 (PD, stimulation of AT $_1$ receptors) for 48 h. Representative blots and the histograms of Western blot analysis of Bax protein (left upper panel), Bcl-2 protein (right upper panel) and activated caspase-3 (left lower panel) in SK-UT-1 cells following stimulation of AT $_1$ and AT $_2$ receptors. Stimulation of AT $_1$ receptors increased the expression of Bax, suppressed the expression of Bcl-2 and considerably increased the cleaved (activated) caspase-3. Activation of Ang II receptors did not alter the LDH release into the media (lower right panel). * $P < 0.05$, ** $P < 0.01$, statistical comparison with vehicle treated cells, calculated by one-way ANOVA followed by a post hoc Bonferroni test for pairwise comparisons. Results are expressed as the means \pm SEM.

mented the expression of the pro-apoptotic Bax protein ($F_{3,20} = 10.259, P < 0.001$) and down-regulated the anti-apoptotic Bcl-2 protein ($F_{3,20} = 3.358, P < 0.05$) via activation of AT₂ receptors. Stimulation of the AT₁ receptor tended to increase the Bax and to decrease the Bcl-2 proteins, the differences failed to reach statistical significance (Figure 3, upper panels). Ang II acting on AT₂ receptors activated caspase-3 (cleavage into 17 and 19 kDa bands) indicating an irreversible progression of apoptotic cascade ($F_{3,16} = 9.125; P < 0.001$, Figure 3, left lower panel). Stimulation of either Ang II receptor did not alter release of LDH into the incubation media (Figure 3, lower right panel). Inhibition of the PPAR_γ by the antagonist, GW 9662, prevented the AT₂ receptor – induced decrease in cell numbers ($F_{2,30} = 8.105, P < 0.01$) and ^{99m}Tc-MIBI uptake ($F_{2,30} = 3.643, P < 0.05$) (Figure 4, upper panels). AT₂ receptor stimulation down-regulated Bcl-2 protein ($F_{2,15} = 4.593, P < 0.05$) and this effect was not reversed by a concomitant treatment with GW 9662 (Figure 4, middle right panel). However, the PPAR_γ antagonist completely prevented the increases in Bax protein ($F_{2,15} = 8.180, P < 0.01$, Figure 4, middle left panel), Bax/Bcl-2 ratio ($F_{2,15} = 16.07, P < 0.001$) and the cleaved caspase-3 ($F_{2,15} = 6.258, P < 0.01$, Figure 4, lower panels), indicating that PPAR_γ activation is a prerequisite for the AT₂-receptor – induced apoptosis in quiescent SK-UT-1 cells.

Cellular localisation of the PPAR_γ and ATIP1 in response to AT₂ receptor stimulation. The localisation and the cellular distribution of the ATIP1 protein and the PPAR_γ are depicted in Figure 5. ATIP1 (Figure 5A, ii/green) is under normal conditions localised in the cell membrane and the cytosol, the PPAR_γ mostly in the cytosol and less in the nuclei (Figure 5A, iii/red and v/violet). Importantly, both proteins were distributed throughout the whole cell. Vehicle treatment did not promote a significant binding of ATIP1 to the PPAR_γ (Figure 5A, iv) and its translocation to the nuclei, as ATIP1 did not matched the distribution of the nuclear fluorescence marker DAPI (Figure 5A, vi). In contrast, SK-UT-1 cells treated with Ang II and losartan showed, firstly, apparent variations in the nuclei size and shape, and secondly, sharply demarcated nuclear localisation of the ATIP1 protein and the PPAR_γ. The ATIP1 protein (Figure 5B, ii/green) formed a complex with the PPAR_γ (Figure 5B, iii/red) (ATIP1/PPAR_γ, Figure 5, iv/yellow) and matched the distribution of the nuclear fluorescence marker DAPI (Figure 5B, i/blue), the spatial overlap of the ATIP1 protein and nuclear signal (DAPI) is depicted in Figure 5B, vi (turquoise). As already mentioned, the PPAR_γ was exclusively localized in the nuclei (PPAR_γ/DAPI, Figure 5B, v/violet). The spatial overlap of the complex ATIP1/PPAR_γ with DAPI in the nuclei is shown in Figure 5B, vii (white) (compare to the image vii in Figure 5A). It is noteworthy to state, that the most intense overlap of the immunofluorescence staining for ATIP1 and the PPAR_γ was observed in nuclei of apoptotic cells (white arrows). Figure 6 shows a time-dependent increase in the ATIP1 protein in the nuclear fraction of SK-UT-1 cells in response to activation of the membrane AT₂ receptors ($P = 0.001$). In contrast, SK-UT-1 cells treated with vehicle

did not show any significant changes in ATIP1 protein. The increases of the ATIP1 protein in response to AT₂ receptor activation detected in cells at 6, 12 and 24 h were statistically significant when compared to the values detected in vehicle-treated controls at the same time points (Figure 6).

Discussion

The present study demonstrates that the effects brought about by activation of Ang II receptors in quiescent SK-UT-1 evolve in time. Stimulation of AT₂ receptors induces differ-

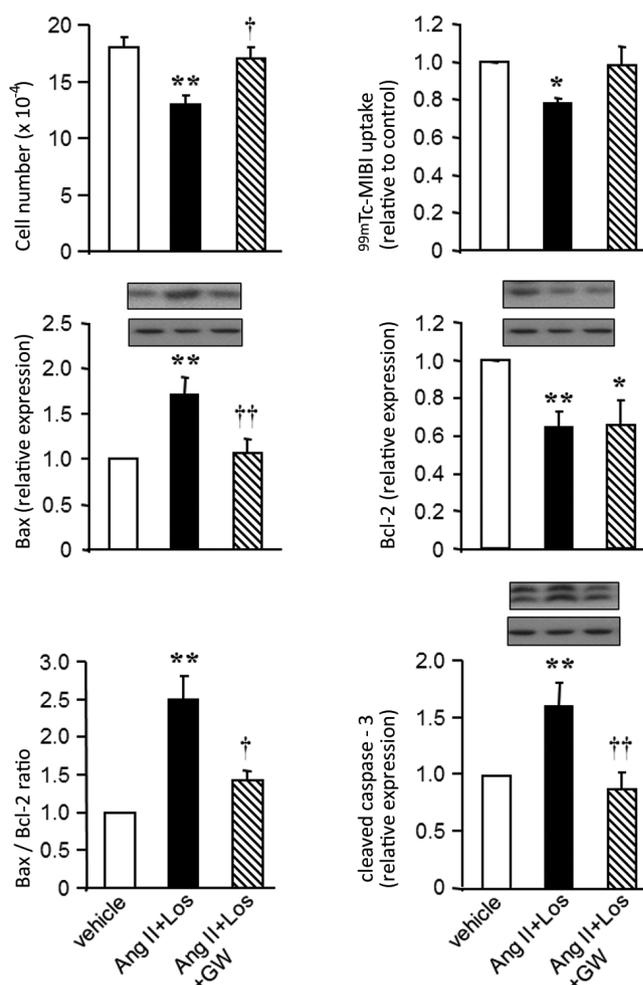


Figure 4. Effects of PPAR_γ inhibition upon the AT₂ receptor-induced apoptosis. The PPAR_γ antagonist, GW 9662 (GW), reversed the effects of AT₂ receptor stimulation on the cell numbers and ^{99m}Tc-MIBI uptake (upper panels). Representative blots and the histograms show that GW 9662 prevented the up-regulation of Bax protein (left middle panel), bax/bcl-2 ratio, and caspase-3 activation (lower panels), indicating a PPAR_γ – dependent mechanism. GW 9662 failed to modify the down-regulation of Bcl-2 protein (right middle panel) in response to AT₂ receptor activation. * $P < 0.05$, ** $P < 0.01$, statistical comparison with vehicle treated cells, and † $P < 0.05$, †† $P < 0.01$ with cells treated with Ang II + losartan (Los), calculated by one-way ANOVA followed by a post hoc Bonferroni test for pairwise comparisons. Results are expressed as the means \pm SEM.

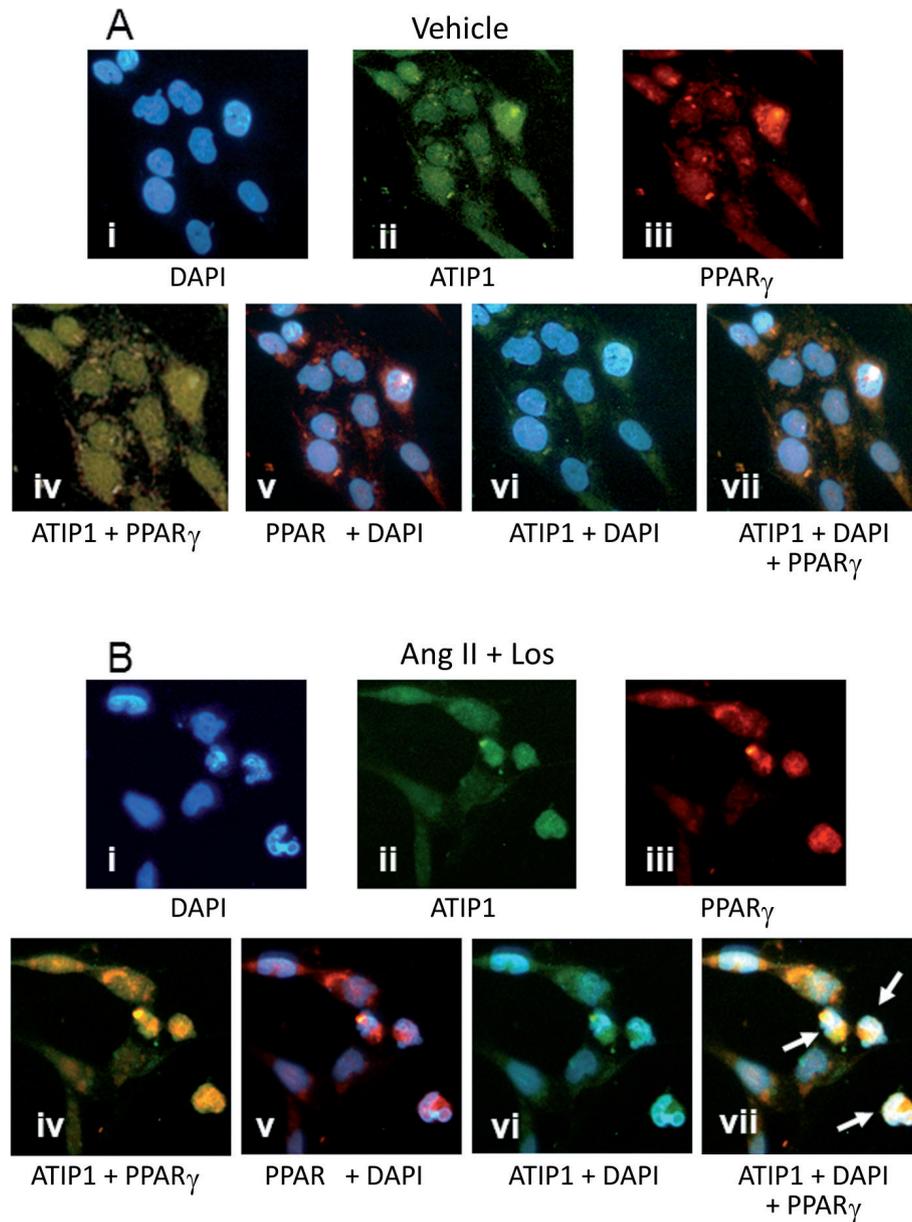


Figure 5. The cellular distribution and co-localisation of ATIP1 with PPAR γ in SK-UT-1 cells treated with vehicle (upper panels, A) and with Ang II and losartan (Los) (selective stimulation of AT $_2$ receptors, lower panels, B). (i) DAPI staining for nuclei (blue), (ii) ATIP1 staining (green) and, (iii) the PPAR γ staining (red). (iv) the co-localisation of ATIP1 with the PPAR γ (yellow), (v) with DAPI (violet), (vi) the co-localisation of ATIP1 with DAPI (turquoise) and (vii) the co-localisation of ATIP1 with the PPAR γ and DAPI. The co-incident signal in the nuclei of apoptotic cells (white arrows) appears white. Notice that in vehicle-treated SK-UT-1 cells, ATIP1 and the PPAR γ were localised in the cytoplasm. Activation of AT $_2$ receptors in SK-UT-1 cells promoted apparent variations in nuclei size and shape and sharply demarcated nuclear localisation of ATIP1 and the PPAR γ .

entiation at very early time points, but reduces cell numbers, promotes cellular damage and activates the intrinsic apoptotic pathway at later time points. The most significant finding is the role of the PPAR γ and its interaction with ATIP1 in mediating the effects initiated by activation of the membrane AT $_2$ receptors.

The AT $_2$ receptor mRNA and protein were reliably detected in SK-UT-1 cells [18]. Ang II is a selective, high affinity ago-

nist for the AT $_2$ receptor ($K_i = 0.63$ nM in the myometrium [22]). A concomitant treatment of cells with Ang II and the AT $_1$ receptor antagonist, losartan, transcriptionally and translationally up-regulates calponin and SM22 α in SK-UT-1 cells by activation of the AT $_2$ receptor. Calponin, a differentiation marker, is highly expressed in differentiated, but down-regulated in de-differentiated SMC cells [23]. Consequently, an up-regulation of calponin rather occurs in de-differentiated,

malignant leiomyosarcoma cells, in which the intracellular level is low [23]. Calponin may act as a tumour suppressor as transfection of calponin into leiomyosarcoma or fibrosarcoma cells suppressed cell proliferation and tumorigenicity [24]. Correspondingly, the calponin induction in leiomyosarcoma cells can be considered as a tumour-suppressive action triggered by the AT₂ receptor.

The mRNA for SM22 α was induced early, up to 1 hour after AT₂ receptor stimulation. To our knowledge, this is the first report demonstrating the effect of AT₂ receptor stimulation on the regulation of SM22 α expression in tumour cells. SM22 α is an early specific marker of SMC differentiation and evidence from experimental studies suggests, that SM22 α acts as a tumour suppressor. A weak or lacking SM22 α expression in de-differentiated cancers derived from SMC supports their proliferation and reduces their sensitivity to apoptosis [see 25 for review]. We therefore propose that the AT₂ receptor-induced differentiation comprising the early induction of calponin and SM22 α is an integral part of anti-proliferative, tumour-suppressive actions of the AT₂ receptor in SK-UT-1 cells. Quiescent SK-UT-1 express the PPAR γ . In the rat pheochromocytoma cells (PC12W cells), the AT₂ receptor-mediated neuronal differentiation was linked to the activation of the PPAR γ [17]. Although the exact mechanism how the membrane AT₂ receptors activate the PPAR γ is not fully understood (see below), the present data point to the role of the PPAR γ signalling in the mediating of the AT₂ receptor-induced differentiation of quiescent SK-UT-1 cells.

AT₁ receptor activation did not alter the cell numbers at 24 h after the onset of the treatment. Although the role of the AT₁ receptor in the initiation and progression of gynaecologic tumours has been well documented [2, 3], this is not a surprising finding, as the AT₁ receptor is down-regulated in quiescent SK-UT-1 cells [18]. Moreover, starvation media are not completely devoid of growth factors and numerous signalling pathways in tumour cells are constitutively activated [26]. Consequently, proliferation-driving effects of growth factors most probably overrode the mitotic activity of the weakly expressed AT₁ receptor. The increased ¹⁸F-FDG incorporation indirectly points to higher mitotic rates triggered by the AT₁ receptor activation and the higher uptake of ^{99m}Tc-MIBI initiated by the AT₂ receptor suggests an accelerated cellular and mitochondrial metabolic activity [19, 20, 27, 28].

As with other cell lines, a 48 h – lasting activation of AT₂ receptors in quiescent SK-UT-1 cells induced apoptosis through activation of the mitochondrial (intrinsic) apoptotic pathway [29-31]. Although the initiation of the apoptotic processes was already observed at earlier time points, the highest apoptotic rates were detected 48 h after the onset of AT₂ receptor activation [18]. We do not have a plausible explanation for the failure of the selective AT₂ receptor antagonist, PD 123177 to completely reverse the AT₂ receptor-induced down-regulation of the anti-apoptotic protein Bcl-2 and the augmented expression of the pro-apoptotic Bax protein. Even when the number of AT₂ receptors considerably increased the

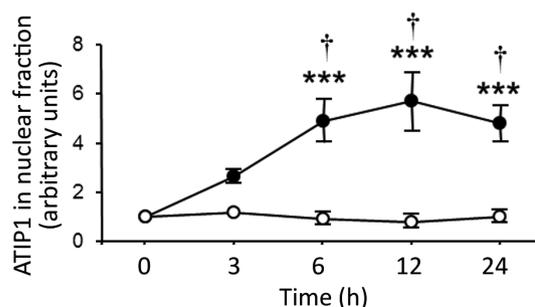


Figure 6. The levels of the ATIP1 protein in the nuclear fraction of vehicle-treated- (empty circles) and AT₂ receptor- activated (black circles) SK-UT-1 cells. Results are expressed as the means \pm SEM. *** P<0.001, statistical comparison to the value detected at the time point 0 (Holm-Bonferroni test). † P<0.05, statistical comparison to the cells treated with vehicle (controls) at the same time point (Wilcoxon-Mann-Whitney-test).

concentration of the antagonist employed in our experiments provided sufficient inhibition. It is conceivable to assume that activation of other than AT₂ receptors also contributed to the observed changes in Bcl-2 and Bax proteins. For instance, The AT₍₁₋₇₎ (*mas*) receptor has been reported to possess anti-tumour activities in lung cancer. Although Ang II does not bind with high affinity to the AT₍₁₋₇₎ receptor, the high-affinity agonist Ang (1-7) can easily be formed from Ang II [32, 33]. Nevertheless, the down-regulation of the anti-apoptotic protein Bcl-2- and the induction of the pro-apoptotic Bax protein promote the formation of Bax homodimers and the activation of the executioner caspase – 3 initiates the irreversible commitment of SK-UT-1 cells to apoptosis. Correspondingly, stimulation of AT₂ receptors reduced the numbers of SK-UT-1 cells and the ^{99m}Tc-MIBI accumulation in SK-UT-1 cells, as the collapse of the mitochondrial membrane potential and mitochondrial activity, which occurs at early stage of ongoing apoptosis, abolished driving forces of ^{99m}Tc-MIBI uptake [20, 34].

Our data provides strong evidence that the AT₂ receptor-induced differentiation and apoptosis are linked to the activation of the PPAR γ . The membrane AT₂ receptor does not couple in a typical manner to G-protein heterotrimers, but binds to multiple AT₂ receptor-interacting/binding proteins (ATIP/ATPB) with distinct intracellular distribution and function [7]. Most of the ATIPs identified to date act as cancer suppressors [7, 9, 35]. ATIP1 is identical with the ubiquitously expressed tumour suppressor protein localized in mitochondria and mimics the inhibitory effects of the AT₂ receptor on cell proliferation. ATIP1, which also regulates the delivery of the AT₂ receptor to the cell membrane and whose expression is up-regulated during quiescence, is therefore the most probable candidate protein to mediate the effects triggered by the membrane AT₂ receptors in SK-UT-1 cells [36, 37]. Very recent findings by Kukida and co-workers [38] have demonstrated that the inhibition of vascular SMC proliferation induced by activation of AT₂ receptors involves ATIP1/ PPAR γ - complex formation.

ATIP1, which is mainly bound to the plasma membrane, was increasingly translocated into the nucleus after AT₂ receptor stimulation. The present results also show a time-dependent accumulation of ATIP1 in the nuclear fraction and an intense co-localisation of ATIP1 and the PPAR γ in nuclei of apoptotic SK-UT-1 cells. This effect was observed only in SK-UT-1 cells treated with Ang II and losartan (selective activation of the membrane AT₂ receptors), but not in vehicle-treated cells. We propose that the dissociation of ATIP1 from the plasma membrane and the subsequent translocation into the nucleus in response to AT₂ receptor stimulation activates the PPAR γ via forming the complex with ATIP1. To our knowledge, this is the first report on the accumulation of ATIP1 in nuclei of cancer cells in response to AT₂ receptor stimulation. The present data suggesting the role of the ATIP1/PPAR γ complex in the activation of the signalling cascades promoting differentiation and/or apoptosis in SK-UT-1 cells upon AT₂ receptor stimulation may initiate a more detailed investigation of cellular processes triggered by the crosstalk between the AT₂ receptor and the PPAR γ in tumour cells.

The current results demonstrate that activation of the membrane AT₂ receptors in quiescent leiomyosarcoma cells exerts anti-tumour effects. AT₁ receptor antagonists are widely prescribed for cardiovascular diseases like hypertension and heart failure. Plasma Ang II concentrations in patients treated with AT₁ receptor antagonists are high and the peptide can increasingly interact with unopposed AT₂ receptors. Therefore, selective AT₁ receptor antagonists, especially telmisartan, which is a partial PPAR γ agonist, have some clinical potential as anticancer drugs in the treatment of human leiomyosarcoma alone or in combination with conventional chemotherapeutics.

Acknowledgments: The authors wish to thank Ms Irina Naujoks and Ms Britta Schwarten for their excellent technical assistance.

References

- [1] FYHRQUIST F, SAIJONMAA O. Renin-angiotensin system revisited *J Intern Med* 2008; 264: 224–236. <https://doi.org/10.1111/j.1365-2796.2008.01981.x>
- [2] INO K, SHIBATA K, KAJIYAMA H, NAWA A, NOMURA S et al. Manipulating the angiotensin system--new approaches to the treatment of solid tumours. *Expert Opin Biol Ther* 2006; 6: 243–255. <https://doi.org/10.1517/14712598.6.3.243>
- [3] DESHAYES F, NAHMIAS C. Angiotensin receptors: a new role in cancer? *Trends Endocrinol Metab* 2005; 16: 293–299. <https://doi.org/10.1016/j.tem.2005.07.009>
- [4] AGER EI, NEO J, CHRISTOPHI C. The renin-angiotensin system and malignancy.
- [5] CUI TX, NAKAGAMI H, NAHMIAS C, SHIUCHI T, TAKEDA-MATSUBARA Y et al. Angiotensin II subtype 2 receptor activation inhibits insulin-induced phosphoinositide 3-kinase and Akt and induces apoptosis in PC12W cells. *Mol Endocrinol* 2002; 16: 2113–2123. <https://doi.org/10.1210/me.2001-0284>
- [6] PICKEL L, MATSUZUKA T, DOI C, AYUZAWA R, MAURYA DK et al. Over-expression of angiotensin II type 2 receptor gene induces cell death in lung adenocarcinoma cells. *Cancer Biol Ther* 2010; 9: 277–285. <https://doi.org/10.4161/cbt.9.4.10643>
- [7] HORIUCHI M, IWANAMI J, MOGI M. Regulation of angiotensin II receptors beyond the classical pathway. *Clin Sci (Lond)* 2012; 123: 193–203. doi: 10.1042/CS20110677. <https://doi.org/10.1042/CS20110677>
- [8] RODRIGUES-FERREIRA S, LE ROUZIC E, PAWLOWSKI T, SRIVASTAVA A, MARGOTTIN-GOGUET F et al. AT2 Receptor-Interacting Proteins ATIPs in the Brain. *Int J Hypertens* 2013; 2013: 513047. doi: 10.1155/2013/513047 <https://doi.org/10.1155/2013/513047>
- [9] RODRIGUES-FERREIRA S1, NAHMIAS C. An ATIPical family of angiotensin II AT2 receptor-interacting proteins. *Trends Endocrinol Metab* 2010; 21: 684–690. doi: 10.1016/j.tem.2010.08.009. <https://doi.org/10.1016/j.tem.2010.08.009>
- [10] LOUIS SN, CHOW L, REZMANN L, KREZEL MA, CATT KJ et al. Expression and function of ATIP/MTUS1 in human prostate cancer cell lines. *Prostate* 2010; 70: 1563–1574. doi: 10.1002/pros.21192 <https://doi.org/10.1002/pros.21192>
- [11] LOUIS SN, CHOW LT, VARGHAYEE N, REZMANN LA, FRAUMAN AG et al. The Expression of MTUS1/ATIP and Its Major Isoforms, ATIP1 and ATIP3, in Human Prostate Cancer. *Cancers (Basel)*. 2011; 3: 3824–3837. doi: 10.3390/cancers3043824. <https://doi.org/10.3390/cancers3043824>
- [12] DESVERGNE B, WAHLI W. Peroxisome proliferator-activated receptors: nuclear control of metabolism *Endocr Rev* 1999; 20: 649–688. <https://doi.org/10.1210/er.20.5.649>
- [13] REN P, ZHANG Y, HUANG Y, YANG Y, JIANG M. Functions of Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) in Gynecologic Disorders. *Clin Med Insights Oncol* 2015; 9: 43–49. doi: 10.4137/CMO.s23527. <https://doi.org/10.4137/CMO.S23527>
- [14] FROHLICH E, WAHL R. Chemotherapy and chemoprevention by thiazolidinediones. *Biomed Res Int* 2015; 2015: 845340. doi: 10.1155/2015/845340. <https://doi.org/10.1155/2015/845340>
- [15] TAKEDA K, ICHIKI T, TOKUNOU T, FUNAKOSHI Y, IINO N et al. Peroxisome proliferator-activated receptor gamma activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells. *Circulation* 2000; 102: 1834–1839. <https://doi.org/10.1161/01.CIR.102.15.1834>
- [16] SUGAWARA A, TAKEUCHI K, URUNO A, IKEDA Y, ARIMA S et al. Transcriptional suppression of type 1 angiotensin II receptor gene expression by peroxisome proliferator-activated receptor-gamma in vascular smooth muscle cells. *Endocrinology* 2001; 142: 3125–3134.
- [17] ZHAO Y, FORYST-LUDWIG A, BRUEMMER D, CULMAN J, BADER M et al. Angiotensin II induces peroxisome proliferator-activated receptor gamma in PC12W cells via angiotensin type 2 receptor activation. *J Neurochem*. 2005; 94: 1395–1401. <https://doi.org/10.1111/j.1471-4159.2005.03275.x>
- [18] ZHAO Y, LÜTZEN U, FRITSCH J, ZUHAYRA M, SCHÜTZE S et al. Activation of intracellular angiotensin AT₂ receptors

- induces rapid cell death in human uterine leiomyosarcoma cells. *Clin Sci (Lond)*. 2015; 128: 567–578. <https://doi.org/10.1042/CS2014062>
- [19] PAUWELS EK, RIBEIRO MJ, STOOT JH, MCCREADY VR, BOURGUIGNON M et al. FDG accumulation and tumor biology. *Nucl Med Biol* 1998; 25: 317–322. [https://doi.org/10.1016/S0969-8051\(97\)00226-6](https://doi.org/10.1016/S0969-8051(97)00226-6)
- [20] MORETTI JL, HAUET N, CAGLAR M, REBILLARD O, BURAK Z. To use MIBI or not to use MIBI? That is the question when assessing tumour cells. *Eur J Nucl Med Mol Imaging* 2005; 32: 836–842. <https://doi.org/10.1007/s00259-005-1840-x>
- [21] UNGEFROREN H, GELLERSEN B, KRULL NB, KALTHOFF H. Biglycan gene expression in the human leiomyosarcoma cell line SK-UT-1. Basal and protein kinase A-induced transcription involves binding of Sp1-like/Sp3 proteins in the proximal promoter region. *J Biol Chem* 1998; 273: 29230–29240. <https://doi.org/10.1074/jbc.273.44.29230>
- [22] LINDMAN S, LINDEBERG G, FRANDBERG PA, NYBERG F, KARLEN A et al. Effect of 3–5 monocyclizations of angiotensin II and 4-aminoPhe⁶-Ang II on AT₂ receptor affinity. *Bioorg Med Chem* 2003; 11: 2947–2954. [https://doi.org/10.1016/S0968-0896\(03\)00212-8](https://doi.org/10.1016/S0968-0896(03)00212-8)
- [23] HORIUCHI A, NIKAIDO T, ITO K, ZHAI Y, ORII A et al. Reduced expression of calponin h1 in leiomyosarcoma of the uterus. *Lab Invest* 1998; 78: 839–846.
- [24] TAKEOKA M, EHARA T, SAGARA J, HASHIMOTO S, TANIGUCHI S. Calponin h1 induced a flattened morphology and suppressed the growth of human fibrosarcoma HT1080 cells. *Eur J Cancer* 2002; 38: 436–442. [https://doi.org/10.1016/S0959-8049\(01\)00390-2](https://doi.org/10.1016/S0959-8049(01)00390-2)
- [25] ASSINDER SJ, STANTON JA, PRASAD PD. Transgelin: an actin-binding protein and tumour suppressor. *Int J Biochem Cell Biol* 2009; 41: 482–486. doi: 10.1016/j.biocel.2008.02.011. <https://doi.org/10.1016/j.biocel.2008.02.011>
- [26] MASON EF, RATHMELL JC. Cell metabolism: an essential link between cell growth and apoptosis. *Biochim Biophys Acta* 2011; 1813: 645–654. doi: 10.1016/j.bbamcr.2010.08.011.
- [27] DELMON-MOINGEON LI, PIWNICA-WORMS D, VAN DEN ABEELE AD, HOLMAN BL, DAVISON A et al. Uptake of the cation hexakis(2-methoxyisobutylisonitrile)-technetium-99m by human carcinoma cell lines in vitro. *Cancer Res* 1990; 50: 2198–1202.
- [28] FURUTA M1, NOZAKI M, KAWASHIMA M, IIMURO M, OKAYAMA A et al. Monitoring mitochondrial metabolisms in irradiated human cancer cells with (99m)Tc-MIBI. *Cancer Lett* 2004; 212: 105–111. <https://doi.org/10.1016/j.canlet.2004.03.002>
- [29] HORIUCHI M, HAYASHIDA W, KAMBE T, YAMADA T, DZAU VJ. Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem* 1997; 272: 19022–19026. <https://doi.org/10.1074/jbc.272.30.19022>
- [30] HORIUCHI M, AKISHITA M, DZAU VJ. Molecular and cellular mechanism of angiotensin II-mediated apoptosis. *Endocr Res* 1998; 24: 307–314. <https://doi.org/10.3109/07435809809032610>
- [31] YAMADA T, HORIUCHI M, DZAU VJ. Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci U S A* 1996; 93: 156–160. <https://doi.org/10.1073/pnas.93.1.156>
- [32] GALLAGHER PE, ARTER AL, DENG G, TALLANT EA. Angiotensin-(1–7): a peptide hormone with anti-cancer activity. *Curr Med Chem* 2014; 21: 2417–2423. <https://doi.org/10.2174/0929867321666140205133357>
- [33] PASSOS-SILVA DG, VERANO-BRAGA T, SANTOS RA. Angiotensin-(1–7): beyond the cardio-renal actions. *Clin Sci (Lond)* 2013; 124: 443–456. doi: 10.1042/CS20120461. <https://doi.org/10.1042/CS20120461>
- [34] VERGOTE J, DI BENEDETTO M, MORETTI JL, AZALOUX H, KOUYOUMDJIAN JC et al. Could 99mTc-MIBI be used to visualize the apoptotic MCF7 human breast cancer cells? *Cell Mol Biol (Noisy-le-grand)* 2001; 47: 467–471.
- [35] DI BENEDETTO M, BIECHE I, DESHAYES F, VACHER S, NOUET S et al. Structural organization and expression of human MTUS1, a candidate 8p22 tumor suppressor gene encoding a family of angiotensin II AT₂ receptor-interacting proteins, ATIP. *Gene* 2006; 380: 127–136. <https://doi.org/10.1016/j.gene.2006.05.021>
- [36] NOUET S, AMZALLAG N, LI JM, LOUIS S, SEITZ I et al. Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT₂ receptor-interacting protein, ATIP. *J Biol Chem* 2004; 279: 28989–28997. <https://doi.org/10.1074/jbc.M403880200>
- [37] WRUCK CJ, FUNKE-KAISER H, PUFE T, KUSSEROW H, MENK M et al. Regulation of transport of the angiotensin AT₂ receptor by a novel membrane-associated Golgi protein. *Arterioscler Thromb Vasc Biol* 2005; 25: 57–64.
- [38] KUKIDA M, MOGI M, OHSHIMA K, NAKAOKA H, IWANAMI J et al. Angiotensin II Type 2 Receptor Inhibits Vascular Intimal Proliferation With Activation of PPAR_γ. *Am J Hypertens* 2016; 29: 727–736. <https://doi.org/10.1093/ajh/hpv168>