

**Effect of the angiotensin AT2 receptor on cell differentiation and apoptosis in
leiomyosarcoma, endothelial cells and PC12W cells**

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Abbreviations

ACE angiotensin I converting enzyme

Ang I angiotensin I

Ang II angiotensin II

Ang III angiotensin (2-8)

Ang IV angiotensin (3-8)

Ang 1-7 angiotensin 1-7

APS ammonium persulfate

AT1 angiotensin II receptor type 1

AT2 angiotensin II receptor type 2

AVP arginine vasopressin

B1 bradykinin receptor type 1

B2 bradykinin receptor type 2

bFGF basic fibroblast growth factor

BK bradykinin

bp base pair(s)

cAMP adenosine 3',5'-cyclic monophosphate

cDNA complementary deoxyribonucleic acid

CEC coronary endothelial cells

cGMP guanosin 3',5'-cyclic monophosphate

CNS central nervous system

DAG diacylglycerol

DEPC diethyl pyrocarbonate

DMEM Dulbecco's minimum essential (or modified Eagle) medium

DMSO dimethylsulfoxide

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTP deoxynucleotide triphosphate

DRGCs dorsal root ganglion cells

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EGF epidermal growth factor

Abbreviations (continued)

ERK early response kinase

FCS fetal calf serum

GAPDH glyceraldehyde-3-phosphate dehydrogenase

G protein guanine nucleotide binding protein

GPCR G protein-coupled receptor

GTC guanidiniumisothiocyanate

GTP guanosin 5'-triphosphate

hr hour

HS horse serum

IGF insulin-like growth factor

IL-1 interleukin-1

IP₃ inositol triphosphate

ITS insulin transferrin selenium

KKS kallikrein-kinin system

MAPK mitogen activated protein kinase

Min minute

MKP-1 MAP kinase-phosphatase-1

MOPS 3-(N-morpholino)propane sulfonic acid

mRNA messenger ribonucleic acid

L-NAME N omega nitro-L-arginine methyl ester

NF-M neurofilament M

NGF nerve growth factor

NO nitric oxide

NOS nitric oxide synthase

NP-40 Nonide P-40

OD optical density

oligo(dT) oligodeoxythymidylic acid

PAA polyacrylamide

PAGE polyacrylamide gel electrophoresis

PBS phosphate-buffered saline

Abbreviations (continued)

PC12W a pheochromocytoma cell line
PCR polymerase chain reaction
PGI₂ prostaglandin
PKC protein kinase C
PLC phospholipase C
PMSF phenylmethanesulfonyl fluoride
POD horse-radish peroxidase
PRCR prolylcarboxypeptidase
RAS renin angiotensin system
RNA ribonucleic acid
RNase ribonucleic acid
rpm rotations per minute
PRCP prolylcarboxypeptidase
RT-PCR reverse transcription-PCR
SAPK stress-activated protein kinase
SDS sodium dodecylsulfate
SHR spontaneously hypertensive rat
SHRSP stroke prone SHR
SMC smooth muscle cells
SNP sodium nitroprusside
TCA trichloroacetic acid
TdT terminal deoxynucleotidyl transferase
TEMED N,N,N',N'-tetramethylethylenediamine
TGF- β transforming growth factor- β
TPCK L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone
Tris-HCl tris(hydroxymethyl) aminomethane hydrochloride
TUNEL TdT-mediated dUTP nick end labeling
Tween 20 polyoxyethylenesorbitan monolaurate
UV ultraviolet
VSMC vascular smooth muscle cells
WKY wistar-kyoto rat
Zfhep zincfinger homeodomain enhancer binding protein

Introduction

General

Angiotensin II (Ang II), an octapeptide that is generated by cleavage from angiotensinogen through an action of two different peptides, renin and angiotensin-converting enzyme (ACE), is the potent effector peptide of the hormonal renin-angiotensin system (RAS) (Skeggs et al., 1956). Ang II exerts a wide range of physiological actions on the cardiovascular, renal and endocrine systems. The peptide plays an important role in the regulation of blood pressure and the maintenance of volume and fluid homeostasis. The classical actions of Ang II include vasoconstriction, facilitation of sympathetic neurotransmission and water and sodium retention, directly or indirectly via aldosterone secretion from the adrenal gland. In the last three decades, evidence has accumulated that Ang II can be formed in various tissues, such as the brain, the kidney, the adrenal gland, the heart and the blood vessels, independently of the hormonal RAS (Campbell, 1987; Dzau, 1988; Unger et al., 1988).

Over the last few years, the classification of angiotensin receptors and our knowledge about their functions, distribution, expression and regulation have been extensively expanded after the development of selective angiotensin receptor ligands. Ang II binds at least to two receptor subtypes, referred to as the AT1 and the AT2 receptor. Both receptors have been cloned; they are seven transmembrane glycoproteins with only 32-34 % homology (de Gasparo et al., 2000; Kambayashi et al., 1993; Murphy et al., 1991). In rodents, but not in humans, the AT1 receptor exists as two isoforms, designated as AT1a and AT1b.

All classical peripheral actions of Ang II are mediated by the AT1 receptor. In addition, this receptor subtype may also initiate proliferation, hypertrophy and growth in various tissues. Although numerous findings have demonstrated that Ang II via the AT2 receptor acts as a modulator of biological programs involved in embryonic development, tissue regeneration and protection, and initiates processes leading to programmed cell death (apoptosis), the physiological and pathophysiological role of the AT2 receptor is still poorly understood (Unger, 1999).

In the recent years, it has become more evident that the RAS plays a major role in the development and the maintenance of arterial hypertension. However, Ang II has

also been implicated in cardiovascular and renal pathology, such as cardiac left ventricular hypertrophy, heart failure, post-infarction remodeling, vascular neointima formation and atherosclerosis (Timmermans et al., 1993).

Historical background on the RAS

The first evidence of the existence of the RAS and its role in the regulation of blood pressure came from the discovery of a pressor principle in 1898 when Tigerstedt and Bergmann injected rabbits with other rabbit kidney extracts. In 1934, Goldblatt and the co-workers rediscovered the importance of renin for blood pressure regulation. They also were able to demonstrate a relationship between renin secretion and renal ischemia (Goldblatt et al., 1934). Within the following years, renin was biologically characterized. In 1941, Page et al identified the renin substrate, angiotensinogen, as a plasma protein and interpreted the RAS as a functional unit. At that time, angiotensin I (Ang I) was considered as the principle hormone causing a direct increase in blood pressure. In 1956, Skeggs et al discovered ACE in the lung, which converts Ang I into Ang II. Ang II was later identified, synthesized isolated and shown to be an octapeptide (Rittel et al., 1957). The angiotensin receptors were identified as a functional entity in 1970 (Lin & Goodfriend, 1970). It was not reported until the end of the 1980s that there are at least two Ang II receptor subtypes: AT1 and AT2 receptors. During the last decades, the studies of the RAS have focused on the wide spectrum of Ang II actions in target tissues, the biological activities of angiotensin-derived peptides and the expression, regulation and function of angiotensin receptors (de Gasparo et al., 2000; Dzau & Gibbons., 1987; Ferrario et al., 1991; Griffin et al., 1991; Peach et al., 1977; Schiavone et al., 1990; Unger et al., 1988; Weber et al., 1995a,b).

Angiotensinogen

Angiotensinogen is a glycosylated α -2 globulin; its molecular weight is between 54 to 60 kDa dependent on species. It is mainly produced in liver and secreted constantly. However, angiotensinogen mRNA can be detected in several tissues; e.g., the brain, the heart, the kidney, the adrenals, the ovaries and the testes (Campbell & Habener, 1986; Cassis et al., 1988; Gomez et al., 1988). The release of angiotensinogen from

the liver is stimulated by bilateral nephrectomy, glucocorticoids, estrogen, thyroid hormone and Ang II (Campbell & Habener, 1986). The level of circulating angiotensinogen can decrease under certain pathological conditions; e.g., liver disease (Ayers, 1967), adrenal insufficiency (Carretero & Gross, 1967) and high sodium intake (Pratt et al., 1989).

Renin

Renin belongs to the family of aspartyl proteases and its amino acid sequence shares structural homology with other members of the family (Blundell et al., 1983; Dhanaraj et al., 1992; Tang et al., 1978). Renin is most active at neutral pH and shows an extremely high substrate specificity for angiotensinogen.

Renin is produced and stored in the juxtaglomerular cells of the kidney. Translation of the renin gene yields preprorenin which is further processed to prorenin and renin (Hsueh et al., 1981; Leckie & McGhee, 1980). Renin is also expressed in other tissues, for instance in the liver, the heart and the blood vessels (Swales & Samani, 1989), the brain (Dzau et al., 1986; Unger et al., 1988), the adrenal cortex (Dzau et al., 1987), the ovary (Kim et al., 1987) and the uterus (Shaw et al., 1989).

It has been found that low, rather than high, plasma renin activity is associated with increased organ damage in hypertension. In contrast, other studies have indicated that low urinary sodium excretion and elevated plasma renin levels are associated with a greater risk of myocardial infarction and increased mortality.

The control of renal renin secretion is quite complex. One of the well established stimuli is the signal from renal afferent arteriolar baroreceptors. Various hormones, including Ang II, arginine vasopressin, prostaglandins and natriuretic peptides participate in the regulation of renin release (Hackenthal et al., 1990). Recent data indicates that nitric oxide (NO) and endothelin can also be involved in the control of renin secretion (Ollerstam et al., 2001).

Renin has been cloned from human (Soubrier et al., 1983), rat (Burnham et al., 1987) and mouse (Rougeon et al., 1981). The renin gene has a high sequence homology with cathepsin D. Humans and rats possess only one gene for renin. In the mouse, two renin genes have been identified, the Ren-1 and the Ren-2 gene. The major difference on the protein level is the presence of glycosylation sites on renin-1 protein.

Angiotensin converting enzyme

ACE is a peptidase with 150 kDa molecular weight. It cleaves the decapeptide Ang I to generate the active Ang II. ACE is mainly localized in the vascular endothelium (Gohlke et al., 1992;). However, the enzyme is widely expressed in other tissues. ACE can also be detected in the epithelial cells of the kidney, in the heart, in the adrenal cortex and medulla, in the brain and the reproductive organs (Chai et al., 1987; Correa et al., 1985; Jackson et al., 1988; Paul et al., 1985; Rix et al., 1981; Skidgel & Erdos, 1987; Yamada et al., 1991). Besides forming Ang II, ACE breaks down bradykinin (BK) into inactive fragments, degrades enkephalins, substance P and angiotensin 1-7 (Ang 1-7) (Chappell et al., 1998; Ehlers & Riordan, 1989; Skidgel et al., 1987; Unger & Gohlke, 1990). Glucocorticoids and the therapy with ACE inhibitors enhance the expression and the activity of ACE (Mendelsohn et al., 1982; Unger et al., 1981).

The conversion of Ang I into Ang II can be effectively blocked by ACE inhibitors. Although ACE inhibitors suppress the activity of the RAS, at least part of their effects can be ascribed to an enhancement of BK effects. The ability of ACE inhibitors to reduce blood pressure due to suppression of Ang II production is extensively used in the treatment of hypertension.

Synthesis of ANG II, the effector peptide of the RAS

The sequence of Ang II is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe in human, horse and pig. Ang II is synthesized by a cascade of enzymatic reactions (Skeggs et al., 1980). In the first step, angiotensinogen is cleaved by renin to form biologically inactive Ang I. Subsequently, ACE converts Ang I into the active octapeptide Ang II by removing of two amino acid residues from the precursor. In this cascade, the formation of Ang I represents the rate-limiting step in the Ang II-synthesis and the major factor, which the activity of RAS depends on.

Ang II is further metabolized to its derivatives. Aminopeptidase A subtracts one amino acid from Ang II to form angiotensin 2-8 (Ang III). Aminopeptidase N splices one amino acid from Ang III to form angiotensin 3-8 (Ang IV) which could be further degraded by a number of enzymes resulting in the formation of small amino acid

fragments. Some of the Ang II-derived metabolites possess biological activity (Ardaillou, 1997; Goodfriend, 1991; Qadri et al., 1998).

Pharmacological characterization of angiotensin receptors

Ang II exerts its actions initially by binding to its receptors. Since the discovery of the non-peptidergic, selective angiotensin receptor antagonists, losartan and PD123319, it has become clear that there are at least two different subtypes of angiotensin receptors, the AT1 and the AT2 receptor (Blankley et al., 1991; Duncia et al., 1990). Both receptors fulfill the criteria, which have been established to identify and characterize distinct receptors. Two other angiotensin receptors (AT3 and AT4) have been proposed, based on operational criteria, but their transduction mechanisms are unknown and they have not yet been cloned.

The AT1 receptor is a seven transmembrane domain receptor and belongs to the G protein-coupled receptor (GPCR) superfamily. Its affinity for Ang II is reduced in the presence of the reducing agent dithiothreitol (DTT) or GTP analogs. The AT1 receptor mediates virtually all of the known physiological actions of Ang II in the peripheral tissue and the brain (de Gasparo et al., 2000). Vascular smooth muscle cells (VSMC) express almost exclusively AT1 receptors which are responsible for Ang II-induced vasoconstriction. Consequently, the selective AT1 receptor antagonists, the so called "sartan" compounds, are successfully used for treatment of hypertension (Unger, 1999).

PD123319 and PD123177 are selective AT2 receptor antagonists since they bind with high affinity to the AT2 receptor. Another pseudopeptidergic compound, CGP42112, is a partial antagonist, since it can also display agonistic effects, at higher concentrations. Like the AT1 receptor, the AT2 receptor also belongs to the family of seven transmembrane domain receptors. The binding of Ang II to the AT2 receptor is not altered by DTT or GTP. The signaling mechanisms of the AT2 receptor are less clearly defined than those of the AT1 receptor.

Until now, the AT4 receptor has eluded molecular characterization. However, recent studies have identified the AT4 receptor to be insulin-regulated aminopeptidase which facilitates memory retention and retrieval in animals after binding with Ang IV (Albiston et al., 2001).

The AT1 receptor gene

The AT1 receptor was successfully cloned in 1991. The human AT1 receptor gene is localized on the chromosome 3 (Curnow et al., 1992; Davies et al., 1994). The AT1 receptor contains 359 amino acids. The amino acid sequence of the human AT1 receptor is 95% identical with those of the rat and bovine AT1 receptors (Bergsma et al., 1992; Furuta et al., 1992). The open reading frame is located on exon 5 (Curnow et al., 1995).

The AT1a and AT1b subtypes found in rats are highly homologous, 95% with respect to the amino acid sequence and 92% to nucleic acids (de Gasparo et al., 2000). The AT1a gene is composed of four exons, the third of which includes the entire 1077-base pair (bp) coding sequence of the receptor protein as well as 5' and 3' untranslated sequences. The gene is mapped on the chromosome 17 (Langford et al., 1992; Murasawa et al., 1993) The rat AT1b receptor gene contains three exons. The open reading frame of the AT1b gene is located entirely in the third exon and the receptor is localized on the chromosome 2 (Tissir et al., 1995). Both AT1 receptor subtypes in the rat have similar pharmacological properties, but their distribution in tissues differ considerably.

AT1 receptor expression and regulation

The AT1 receptor is present in all tissues where Ang II is known to exert its classical effects. In human, AT1 receptors can be found in various tissues. The AT1 receptor is highly expressed in VSMC, the kidney, the uterus, the heart, the lung, the brain, and in the urinary tract (Ahmed et al., 1995; Allen et al., 1999; Mifune et al., 2001; Miyazaki & Ichikawa, 2001; Paradis et al., 2000; Vinson et al., 1995). In adult rodents, AT1a receptors are expressed in all of these tissues. AT1b receptors have been mainly identified in the adrenal and pituitary glands, some brain regions and the testis. VSMC contain only the AT1a receptor subtype (Burson et al., 1994; Gasc et al., 1994; Timmermans et al., 1992; Vinson et al., 1995).

The presence and numbers of AT1 receptors vary considerably among different tissues. The patterns of the AT1 receptor distribution suggests that this angiotensin receptor subtype exerts very important biological activities in these tissues. The

expression of the AT1 receptor can be affected upon different pathological conditions, for instance in renovascular hypertension, after myocardial infarction, or bilateral nephrectomy, during the process of heart remodeling (Iwai & Inagami, 1992; Llorens-Cortes et al., 1994; Nio et al., 1995; Yamamoto et al., 2000; Zhu et al., 2000).

Treatment with glucocorticoids dramatically increases the expression of both types of AT1 receptors in the rat heart (Della Bruna et al., 1995). Estrogen treatment of rats was demonstrated to suppress the expression of AT1b mRNA level in the pituitary gland and to elevate the AT1a mRNA levels in smooth muscle cells (SMC) (Kakar et al., 1992; Nickenig et al., 1996). Other forms of a hormonal regulation of the AT1 receptor expression have been reported, such as the insulin-induced up-regulation of AT1 receptors in VSMC. Progesterone, on the other hand, down-regulates the number of AT1 receptors in VSMC (Nickenig et al., 1998; Nickenig et al., 2000;). NO decreases, whereas epidermal growth factor (EGF) enhances the AT1a receptor gene expression in VSMC (Guo & Inagami, 1994, Ichiki et al., 1998). Ang II itself is able to down-regulate AT1 receptors in different cell types, such as in VSMC, mesangial cells and adrenal fasciculate reticularis cells (Ichiki et al., 2001; Makita et al., 1992; Naville et al., 1993).

AT1 receptor signaling pathways

The AT1 receptor signaling mechanisms have been intensively studied and the signaling pathways of the AT1 receptors are now largely well understood (de Gasparo et al., 2000). As a member of GPCR family, the AT1 receptor seems to couple mainly to G_q initiating the activation of phospholipase C (PLC) leading to phosphatidylinositol hydrolysis to form inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 evokes a subsequent increase in intracellular calcium, which mediates several of the effects regulated by the AT1 receptors, such as vascular smooth contraction, secretion of aldosterone from the adrenal cortex cells (de Gasparo et al., 2000).

Other G proteins, reported to mediate the AT1 receptor-induced effects, are the $G_{12/13}$ family (leading to L-channel activation) and $G_{i/o}$ proteins (which inhibit adenylyl cyclase). In addition to these pathways, the binding of Ang II to its AT1 receptor also

leads to the phosphorylation of a number of downstream mediators like PLC γ or pp60^{c-src} (de Gasparo et al., 2000).

Recently, protein kinase C (PKC) beta subtype, which is stimulated by DAG, has been added to the array of AT1 receptor induced signaling. Most of these signaling cascades can finally result in the activation of inducible transcription factors such as c-Fos, c-Jun or KROX belonging to the DNA-binding proteins that regulate the expression of genes at the transcriptional level and thus transform the AT1 receptor-mediated signal into cellular function (Blume et al., 1999).

In addition, the AT1 receptor may play a role in the activation of mitogen-activated protein kinases (MAP kinases) as has been demonstrated in cardiac myocytes (Yamazaki et al., 1999). MAP kinases are serine/threonine protein kinases which are activated in response to growth factors and likely involved in the cell development and differentiation. After their stimulation, they translocate into the nucleus of the cells to stimulate nuclear transcription factors involved in the proliferation, differentiation, apoptosis (Ahn et al., 1990; Bucher et al., 2001).

AT1 receptor can activate the JAK-STAT pathway and Jun-Kinase, other members of the MAP kinase family, to transduce cell surface signals into the cell cytoplasm and nucleus where they bind to specific DNA elements resulting in the transcription of early growth response genes, such as c-fos which lead to cell proliferation similar to cytokine receptors (Bhat et al., 1994; Blume et al., 1999; Leaman et al., 1996; Marrero et al., 1995). These findings provide very strong support for the role of the AT1 receptor in cellular growth through cross-talk with more than one tyrosine kinase systems.

The AT2 receptor gene

The AT2 receptor has been cloned from mouse, rat and human (Ichiki et al., 1994; Koike et al., 1995; Martin et al., 1994; Nakajima et al., 1993; Tsuzuki et al., 1994). In all three species, the AT2 receptor cDNA encodes for a 363 amino acid receptor. The sequence comparisons reveal a high homology on the nucleic acid as well as the amino acid levels (89% and 91%, respectively). The genomic DNA of all three species consists of three exons and its entire coding sequences are located in the third exon. The AT2 receptor genes in human, rats and mice reside as a single copy on the X-chromosome (Koike et al., 1994). Comparisons of the AT1 and AT2 nucleic

acid sequences reveal only a poor, 34% homology. A complete divergence between AT1 and AT2 receptors is seen in the third intracellular loop, even more extensive differences have been found in the carboxyl terminal tail.

The AT2 receptor protein contains 5 potential glycosylation sites in its extra-cellular N-terminal tail. In addition, there is a potential PKC phosphorylation site in the second intracellular loop. Furthermore, there are 3 consensus sequences for phosphorylation by PKC and one phosphorylation site by cyclic adenosine 3',5' monophosphate (cAMP)-dependent protein kinase in the c-terminal tail of the receptor in the cytoplasm (de Gasparo et al., 2000).

AT2 receptor expression and regulation

Compared to AT1 receptors, AT2 receptors are differently distributed in tissues. The AT2 receptor is predominantly expressed in fetal tissues. After birth, the ratio of AT1 and AT2 receptor expression is reversed with the AT1 receptor subtype being the dominant one in most of the tissues of the adult organism (Unger, 1999). In the adult organism, both subtypes of angiotensin receptors can be found in the adrenal gland and vascular endothelial cells, the kidney and the heart, whereas the AT2 receptor predominates in the uterus, ovarian granulosa cells and some brain areas (Obermüller et al., 1991; Stoll et al., 1995; Unger, 1999). In the myometrium, the AT2 receptor is predominantly expressed under non-pregnant conditions, the number of AT2 receptors declines during pregnancy, but returns to the non-pregnant level after parturition (de Gasparo et al., 1994).

Although the AT2 receptor is not a common angiotensin receptor in a number of tissues in adult organisms, its expression can be dramatically increased under pathological conditions such as vascular injury, myocardial infarction, congestive heart failure, renal failure, brain ischemia, nerve transection (Unger, 1999).

An up-regulation of the AT2 receptor expression has been also observed after treatment with interleukin-1 β (IL-1 β), insulin, insulin-like growth factors (IGF), sex hormones. glucocorticoids, norepinephrine and growth factors down-regulate the AT2 receptor expression (Mancina et al., 1996; Matsubara & Inada, 1998; Stoll et al., 2002; Unger, 1999). Thus, it appears that a number of factors, particularly growth factors and hormones, can influence the expression of the AT2 receptor in various tissues (Stoll & Unger, 2001).

AT2 receptor signaling pathways

The elucidation of the AT2 receptor-coupled intracellular signaling pathways turns out to be much complicated than in the case of the AT1 receptor. Although the AT2 receptor has structural features commonly shared by seven transmembrane receptors, it does not reveal all functional features commonly attributed to this class of receptors. As stated below, the intracellular signaling pathways described for the AT2 receptor so far depend mostly on the type of the cell/tissue examined and employed experimental conditions.

In contrast to the AT1 receptor, many studies on the signaling of the AT2 receptor have been performed in cells of neuronal origin (PC12W and NG 108-15 cell lines). Some features of the AT2 receptor make it unique among GPCR (Nouet & Nahmias, 2000). G_i proteins seem to be the G protein subtype which may couple to the AT2 receptor. A variety of signaling mediators have been characterized, with partly contradictory results. Thus, the AT2 receptor has been shown to mediate an increase as well as a decrease of intracellular cyclic guanosine 3', 5'-monophosphate (cGMP), an activation or deactivation of early response kinase 1/2 (ERK 1/2) MAP kinases and an up- or down-regulation of phosphatases PP2A, MKP-1, SHP-1. The nature of the signal transduction pathways for this receptor is obviously to a high degree dependent on the total signal input into the cell at the time of the AT2 receptor stimulation. Thus, stimulation with Ang II leads to an early AT2 receptor mediated increase in ERK1/2 activity in PC12W cells; in contrast, a nerve growth factor (NGF)-stimulated ERK1/2 activation in the same cells is later inhibited by AT2 receptor activation (Stroth et al., 2000). Other signal transduction molecules stimulated by AT2 receptor activation are particulate guanylate cyclase, T-type Ca^{2+} and K^+ currents, NO release and activation of phospholipase A2, the latter resulting in the release of arachidonic acid and its metabolites and ultimately in the activation of the Ras kinase/MAP kinase pathway (de Gasparo et al., 2000). More recently, *in vivo* studies have shown that the AT2 receptor-induced release of arachidonic acid may contribute to the activation of Na^+/HCO_3^- symporter system, which regulates intracellular pH. Thus, the AT2 receptor seems also to be involved in the processes regulating intracellular pH following injury (Haithcock et al., 1999; Kohout & Rogers., 1995). As already mentioned above, activation of guanylate cyclase by the AT2

receptor increases cellular cGMP levels, NO production and its release (Gohlke et al., 1998; Liu et al., 1997; Siragy & Carey, 1996; Wiemer et al., 1993).

It has been demonstrated recently, that Ang II acting on the AT₂ receptor can induce apoptosis. The apoptosis-promoting properties of the AT₂ receptor have been shown to involve ceramide generation as well as activation of caspase 3 (Dimmeler et al., 1997; Gallinat et al., 1999; Lehtonen et al., 1999) (see below).

The effect of Ang II on cell proliferation/apoptosis and differentiation may be, at least partly, attributed to the AT₂ receptor-induced stimulation on MAP kinase activity (Horiuchi et al., 1998; Lehtonen et al., 1999; Stroth et al., 2000). Recent studies in VSMC indicate that the AT₂ receptor activates not only the ERK MAP kinases but also Janus kinases and signal transducers and activators of transcription (Jak/STAT) which seem to be the key elements of signaling pathways for growth factors and cytokines to stimulate the growth of these cells (Stoll & Unger, 2001).

Recently, our group has identified a zincfinger homoeodomain enhancer binding protein gene (*Zfhep*) encoding for a transcription factor, which is expressed after activation of the AT₂ receptor in cells of neuronal and vascular origin. *Zfhep* may represent a link between the AT₂ signaling and downstream events involved in proposed effects of the AT₂ receptor in the development and regeneration (Stoll et al., 2002).

Local renin angiotensin systems (RASs)

In addition to the systemic RAS, the existence of tissue RASs has been established. The tissue RASs express all key RAS components, notably angiotensinogen and renin, independently of the circulating RAS. The existence of local RAS has been demonstrated in various tissues and cell lines such as in the pancreas, the brain, the adrenal gland, the heart blood vessels, the uteroplacental unit, the human uterine decidual cells (Bader et al., 2001; Leung & Carlsson, 2001; Li et al., 2000; Nielsen et al., 2000; Sigmund, 2001). Tissue RASs have been implicated; e.g., in the local regulation of blood flow, angiogenesis, proliferation and differentiation of cells, inflammation and tissue remodeling independently of systemic RAS. For instance, the RAS in the brain participates in the regulation of blood pressure, drinking behavior, natriuresis, secretion of pituitary hormones (Culman et al., 2001). A local RAS in adipose tissue has also been identified. Interestingly, this RAS has been

implicated in the pathogenesis of hypertension and some obesity-related disorders in humans (Engeli et al., 1999).

A number of experiments have been carried out to characterize the Ang II receptors in the uterus and to study the regulation of their expression in different species. The human non-pregnant uterus expresses the AT₂ receptor at a high proportion as compared to the AT₁ receptor. Similarly, in the myometrium of porcine uterus, the AT₂ receptor is highly expressed; its expression is, however, down-regulated during gestation. Species dependence exists, as only 40% and 60% of Ang II-binding sites are AT₂ receptors in the rat and rabbit uterus, respectively (de Gasparo et al., 2000). In contrast, the AT₁ receptor is expressed in the myometrium independently of the gestation (Nielsen et al., 1997). The expression of the AT₂ receptor in the human myometrium varies dramatically during the menstrual cycle (Mancina et al., 1996).

Regulatory functions of the RAS

RAS and cardiovascular system

Ang II regulates blood pressure through peripheral and central mechanisms. In the periphery, Ang II directly induces vasoconstriction by activating of AT₁ receptors located on VSMC and causes sodium retention in the kidney through stimulation of aldosterone release from the zona glomerulosa in the adrenal gland and by a direct tubular mechanism. Ang II via the AT₁ receptor facilitates peripheral noradrenergic neurotransmission by augmenting norepinephrine release from sympathetic nerve terminals and by enhancing the vascular response to norepinephrine (Balt et al., 2001). Ang II also facilitates vasoconstriction by stimulation of the catecholamine release from the adrenal gland (Peach et al., 1966). This effect can be attributed to Ang II circulating in blood as well as to the peptide synthesized locally in the adrenal tissue. Enhanced expression of endothelin-1 in response to Ang II may further contribute to the vasoconstrictor effects of Ang II (Hahn et al., 1993). While the AT₁ receptor induces vasoconstriction and increases the blood pressure, the AT₂ receptor exerts opposite effects. AT₂-null mice show a slight elevated blood pressure which further increases during Ang II infusion (Siragy & Carey, 1999).

In addition to the peripheral effects, Ang II in the brain regulates blood pressure through the release of vasopressin modulation of the sympathetic activity and the

attenuation of the baroreceptor reflex. These effects are mediated by central AT1 receptors (Höhle et al., 1995). Additionally to these effects, Ang II acting on periventricular and hypothalamic areas induces an immediate drinking response. The peptide is also involved in the regulation of the release of a number of hormones from the pituitary gland into the circulation (Saavedra, 1992).

In the periphery, Ang II can induce heart- and vascular hypertrophy independently on its pressor mechanisms. Treatment of spontaneously hypertensive rats (SHR) with sub-antihypertensive doses of ACE inhibitors results in a decrease in cardiac hypertrophy (Baker et al., 1990). Studies in our laboratory have also demonstrated a regression of the left ventricular mass in SHR treated with sub-antihypertensive doses of ramipril, an ACE inhibitor (Gohlke et al., 1996; Gohlke et al., 1994). These findings clearly indicate that Ang II directly promotes cardiac growth independently of its haemodynamic effects.

The AT2 receptor has been implicated in events associated with cardiac remodeling after myocardial infarction. Increased expression of this angiotensin receptor subtypes has been observed after myocardial infarction or in failing heart (Matsumoto et al., 2000; Tsutsumi et al., 1998; Zhu et al., 2000).

RAS and growth, proliferation and differentiation

High expression of the AT2 receptor during embryogenesis indicates that Ang II may participate in the regulation of both, cell proliferation and differentiation. The low expression of the AT2 receptor in early embryonic stages but its very high levels during late embryonic development and rapid decline in the neonatal period suggest a role for the AT2 receptor in fetal vascular development in late embryonic and early postpartum development (Shanmugam et al., 1995; Viswanathan et al., 1991).

Evidence for an antiproliferative effect of Ang II through the activation of its AT2 receptors first came from the study in coronary endothelial cells (CEC) (Stoll et al., 1995) and have been confirmed later, in NG108-15 cells, PC12W cells, R3T3 cells, renal mesangial cells (Goto et al., 1997; Laflamme et al., 1996; Meffert et al., 1996; Munzenmaier & Greene, 1996; Tsuzuki et al., 1996).

In VSMC, which express only AT1 receptors in culture, Ang II exerts a growth-promoting effect. However, in cells transfected with the AT2 receptor, the proliferation is reduced and MAP kinase activity is inhibited. Similarly, in NIE-115 cell line, the AT2

receptor inhibits the activity of MAP kinases and suppressed thus the cell growth (Bedecs et al., 1997). Similar effects have been observed in fibroblasts, PC12W cells and neuronal cells (Huang et al., 1996; Meffert et al., 1996; Yamada et al., 1996).

In vivo experiments employing transfection of an AT2 receptor expression vector into the balloon-injured rat carotid artery demonstrate that the over-expression of the AT2 receptor attenuates the neointima formation (Nakajima et al., 1995). This finding indicates that the RAS has the possibility to balance via activation of AT2 receptors the growth response elicited by Ang II through the AT1 receptor.

The stimulation of PC12W cells, which express exclusively the AT2 receptor, stimulation with NGF is extensively used as a model to study neuronal differentiation. Treatment of PC12W cells with NGF up-regulates protein filaments, like microtubules, actin filaments and intermediate filaments which play a crucial role in the stability and function of neuronal PC12W cells. AT2 receptor activation by daily treatment of the PC12W cells with Ang II not only directly promotes neuronal differentiation, as evidenced by augmented neurite outgrowth and by up-regulation of polymerised tubulin, but also significantly enhances the NGF-mediated morphological differentiation (Meffert et al., 1996). Comparison of the effects of NGF on tubular and neuroskeleton proteins with those of the AT2 receptor reveals similarities; e.g., the up-regulation of β -tubulin, but also conspicuous differences. Whereas NGF up-regulates neurofilament M (NF-M), a cell differentiation marker, the AT2 receptor not only down-regulates this protein but also inhibits the NGF-induced up-regulation of this neurofilament in PC12W cells (Gallinat et al., 1997). These results suggest that the NGF-induced mechanisms leading to differentiation in PC12W cells are distinct from those induced by Ang II via the AT2 receptor. Similar effects have been observed in the neuronal cell line NG108-15 (Laflamme et al., 1996).

RAS and tissue regeneration

In general, the process of wound healing and tissue repair is controlled by a variety of mechanisms. Recent findings indicate that Ang II may interfere with these processes as increased tissue levels of the AT2 receptor have been observed after skin injury or after myocardial infarction (Unger, 1999). Since it has become apparent that Ang II acting through the AT2 receptor plays a role in wound healing and repair of peripheral tissues, the attention of investigators has turned to the role of Ang II in

processes occurring during regeneration after injury of neuronal tissue. Adult neurons usually do not re-innervate their target regions after injury, probably due to absence of requisite neurotrophic agents. However, if they are supplied with growth-promoting substances, they are able to generate new processes over long distances and, eventually, re-innervate their target region. Stimulation of AT2 receptors in PC12W cells down-regulates NF-M as it occurs in nerve fibre regeneration (Gallinat et al., 1997). Reduced neuronal expression of this protein has been found following nerve transection (Lucius et al., 1998). Following sciatic nerve transection, a several-fold up-regulation of mRNA coding for the AT1 and AT2 receptors is demonstrated in dorsal root ganglion neurons and in sciatic nerve segments proximal and distal to the cell body. Sciatic nerve crush results in a time-dependent up-regulation of AT2 receptor mRNA levels in sciatic nerve segments which coincides with the successful regeneration of nerve fibres (Gallinat et al., 1998). These findings indicate that the AT2 and AT1 receptor-mediated pathways are involved in Schwann cell-mediated myelination and in the control of neuroregenerative responses in the peripheral nervous system.

Ang II-mediated effects have also been investigated in axonal regeneration of postnatal rat retinal explants and cultured dorsal root ganglion cells (DRGCs). In the *in vitro* model of postnatal retinal explants and cultured DRGCs, which are comparable with adult, non-regenerating DRGCs, as well as in the *in vivo* model of optic nerve crush, Ang II induces a concentration-dependent outgrowth of neurites. These effects of Ang II, both *in vitro* and *in vivo*, are mediated by the AT2 receptor. Like in the previous studies carried out on peripheral nerves, the regeneration process is paralleled by a time-dependent increase in AT2 receptor mRNA expression in the retina and the crushed optic nerve (Lucius et al., 1998). These findings point to a role of Ang II and its receptors in the regeneration processes occurring in neuronal tissue following injury and provide a direct evidence that stimulation of AT2 receptors promotes axonal regeneration not only *in vitro*, but also under *in vivo* conditions after neuronal lesion.

RAS and apoptosis

Apoptosis, is a prominent feature of normally developing tissues, as well as tissues that have undergone injury or damage. The intricate balance between pro- and anti-apoptotic proteins determines whether the cell will undergo apoptosis or survive. Several studies have reported that increased AT₂ receptor expression in a developing fetus correlates with extensive apoptosis (Chamoux et al., 1999).

Neurons undergo apoptosis after ischemic insult or when they are seriously injured; e.g., after axotomy. The risk of apoptosis and the potency for axonal degeneration are closely related: lesions occurring close to the neuronal cell bodies initiate a potent cell body response directed towards regeneration, but simultaneously, the risk of apoptosis is high. Neuron lesion more distally usually do not undergo apoptosis but the regeneration processes are weak. These observations suggest that neuronal injury or damage initiates a series of molecular events which are identical for both, regeneration and apoptosis. Generally, the classical MAP kinase cascades, like the ERK1/2 pathway, the stress-activated protein kinase (SAPK) cascade (JNK pathway) and the p38 pathway have been implicated in apoptosis. ERK1 and ERK2 kinases inhibit apoptosis whereas SAPK and p38 act pro-apoptotic. It is likely that a dynamic balance between the SAPK cascades and MAP kinases plays a critical role in cell survival and death (Kinloch et al., 1999).

Recent findings indicate that Ang II acting on its receptors not only exerts growth promoting effects or promotes neuronal differentiation and regeneration, but is involved in the regulation of cellular pro- and anti-apoptotic events. It has been demonstrated that the AT₂ receptor exerts growth inhibitory and pro-apoptotic effects by antagonising the effects mediated by the AT₁ receptor or growth factors in various cell lines, like VSMC, neuronal PC12W cells or fibroblasts. Stimulation of the AT₂ receptors in serum-deprived PC12W cells is reported to induce apoptosis (Yamada et al., 1996). In these cells, NGF inhibits the internucleosomal DNA fragmentation induced by serum deprivation, whereas Ang II upon binding to AT₂ receptors antagonises the NGF-mediated survival and induced apoptosis. The pro-apoptotic events mediated by the AT₂ receptor include activation of tyrosin phosphatases such as MKP-1 and inactivation of MAP kinase (ERK1/2) which results in Bcl-2 protein dephosphorylation (inactivation of an anti-apoptotic factor) and up-regulation of the pro-apoptotic Bax protein (Horiuchi et al., 1998; Horiuchi et al., 1997). Analysis of

functional domains of the AT2 receptor in neuronal PC12W cells has revealed that the intermediate portion of the intracellular third loop is important for the apoptotic effects mediated by this receptor (Lehtonen et al., 1999). The axotomy-induced AT2 receptor up-regulation associated with the down-regulation of neurofilament M (NF-M) can also be interpreted in terms of apoptosis rather than neuronal regeneration (Gallinat et al., 1997). Ceramides, which are generated by a PLC type reaction from the precursor sphingomyelin, serve as mediators of the cellular responses to a variety of apoptotic stimuli. The molecular mechanisms responsible for the ceramide-induced cell death involve in activation of stress kinases or caspases (Hofmann & Dixit, 1998). We have recently demonstrated that stimulation of the AT2 receptors selectively induces de novo synthesis of ceramides in PC12W cells (Gallinat et al., 1999). The AT2 receptor-induced ceramide accumulation precedes the onset of caspase 3 activation and DNA fragmentation (Lehtonen et al., 1999). An interesting new aspect of AT2 receptor signaling has been shown recently in several cell types: the ligand-independent signaling of the AT2 receptor. An over-expression of the receptor without binding of Ang II is sufficient to induce apoptosis (Miura & Karnik, 2000).

Links between the kallikrein-kinin system and the RAS system

The plasma kallikrein-kinin system (KKS) has been traditionally linked to physiologic hemostasis. In the recent years, the link between the BK and NO production and NO-mediated effects has increasingly attracted the attention of scientists (Tschöpe et al., 2002). The endothelial cell-associated active kallikrein cleaves the high-molecular weight kininogen to form BK, which can then activate its B2 receptor. Activation of this receptor regulates vascular tone by stimulating NO formation in endothelial cells (Schmaier, 2002). The B2 receptor knock-out mice is a very useful model to study the physiological relevance of the KKS and the interaction between this system and the RAS. These mice develop mild hypertension, cardiac hypertrophy, chamber dilatation and myocardial damage, elevated left ventricular and end-diastolic pressure, and they show exaggerated vasopressor response to Ang II. In the presence of Ang II, these mice have increased blood pressure and reduced renal blood flow (Emanuelli et al., 1999; Madeddu et al., 1999). ACE and prolylcarboxypeptidase (PRCP) are the key molecules which interfere with both

systems. As already mentioned, ACE converts inactive Ang I to the vasoconstrictor peptide Ang II. The same enzyme also degrades BK to BK₍₁₋₇₎ and BK₍₁₋₅₎. PRCP is the enzyme which degrades Ang II to the vasodilating peptide Ang (1-7). This peptide also stimulates NO and prostaglandin (PGI₂) formation which potentate the effects of BK. PRCP has also the ability to convert prekallikrein to kallikrein, which cleaves kininogen to liberate BK. Thus, PRCP is the enzyme which degrades the vasoconstrictor peptide Ang II and forms the vasodilator BK and Ang (1-7). BK stimulates NO and prostaglandin PGI₂ formation, thus counterbalancing the vasoconstrictor effects of Ang II (Fig 1) (Schmaier, 2002).

ACE inhibitors have been successfully introduced in the treatment of hypertension and other cardiovascular diseases. The improvement of cardiac, renal and endothelial functions observed after treatment with ACE inhibitors appears to be, at least partly, B2 receptor-mediated, as evidenced by an increased NO production and their reversal by B2 receptor antagonism (Cachofeiro et al., 1995; Linz et al., 1995). AT1 receptor antagonists have been shown to produce similar effect like ACE inhibitors with respect to the improvement of cardiac, vascular and renal functions. The underlying mechanism seems to involve the AT2 receptor. When AT1 receptors are blocked, Ang II can increasingly interact with AT2 receptors, as AT1 receptor antagonists leave the AT2 receptor unopposed and rather expose it to elevated Ang II levels (Carey et al., 2001; Sosa-Canache et al., 2000). There is only indirect evidence pointing to a link between the AT2 receptor and the BK-system. Experiments employing bovine endothelial cells, rat carotid arteries, canine coronary arteries or rat kidney have demonstrated that Ang II can stimulate NO production, and this effect is, at least partly, mediated by BK (Boulanger et al., 1995; Seyedi et al., 1995; Wiemer et al., 1993). These findings indicate that Ang II acting on the AT2 receptor can enhance the production of BK and NO and thus indirectly interfere with the regulation of BK-mediated responses (Siragy et al., 1999). Moreover, activation of AT1 receptors triggers the formation of reactive oxidant species which play a role in the pathophysiology and progression of cardiovascular diseases (Ichiki et al., 2001). Stimulation of AT2 receptors has opposite effects since augmented NO generation due to increased BK production neutralises superoxide anions (Tsutsumi et al., 1999).

Several recent studies have also suggested a link between the BK/NO system and organ protection. As already stated, BK through its B2 receptor triggers a cascade of

events, comprising liberation of PGI₂ and NO and stimulation of soluble guanylate cyclase to form cGMP (Higashida et al., 1999). Recent data from our group indicate that Ang II via its AT₂ receptor increases aortic cGMP levels in the aorta of SHR, in bovine aortic endothelial cells and in PC12W cells. Both, the activation of NO synthase and the stimulation of the B₂ receptor, are involved in the AT₂ receptor-mediated Ang II effect on cGMP levels (Gohlke et al., 1998).

PC12W, CEC and SK-UT-1 cells: models to study the functions of the AT₂ receptor

AT₂ receptors are highly expressed in a number of cell lines, like pheochromocytoma cell line (PC12W cells), Swiss mouse 3T3 fibroblasts (R3T3 cells) and mouse neuroblastoma cell line (NG108-15). In general, these cells express exclusively AT₂ receptors, but not AT₁ receptors up to certain cell passages, and are, therefore, very useful to study the regulation, signal transduction pathways, function and (patho)physiological relevance of the AT₂ receptor.

Uterus leiomyosarcoma, a type of smooth muscle neoplasm of the uterus, is extremely malignant with high rates of local recurrence and metastasis to distant sites. SK-UT-1 is a human uterus leiomyosarcoma cell line derived from a human mixed malignant mesodermal tumor of the uterus obtained from a single biopsy of the sarcoma. It belongs to myometrial cell lines. This cell line has a higher number of insulin-like growth factor (IGF) II receptors than insulin- and IGF I receptors. IGF-II is highly potent in stimulating the proliferation of these cells, IGF-I has, on the contrary, no effect. Insulin can stimulate the DNA synthesis only at higher, pharmacological concentrations (Nagamani & Stuart, 1996). TC21 is the fourth member of the ras gene family and the mutation of this gene is related to uncontrolled proliferation of these cells. A mutation comprising an insertion of nine base pairs at TC21 codon 24 could be detected in SK-UT-1 cells (Huang et al., 1995). It has been found that all cAMP-dependent as well as some basal biglycan, a prototype member of the proteoglycan family, negative feedback loops regulating transforming growth factor (TGF)- β , transcription in SK-UT-1 are mediated through activated protein kinase A (Ungefroren et al., 1998).

Coronary endothelial cells, primary cells isolated from the rat coronary artery, are characterized by their cobblestone morphology and the presence of the receptors for

acetylated LDL (Voyta et al., 1984). The purity of the cell culture must be verified by staining with a monoclonal antibody for smooth muscle α -actin to exclude pericyte contamination. These cells have both Ang II receptor subtypes: the AT1 and AT2 receptor (Stoll et al., 1995).

The PC12W cell line is a sub-strain from a clonal isolation of a rat adrenal chromaffin cell tumor (Greene and Tischler, 1976). PC12W cells exclusively express AT2 receptors at lower, less than 18, cell passages. Most of the studies on the signaling pathways of the AT2 receptor have been performed in these cells. Experimental results obtained from this cell line might be representative of the AT2 receptor functions in neuronal tissues. These tumor cells of neuronal origin are widely used to study neurotransmitter synthesis and release, ionic channels and mechanisms of actions of growth factors, like NGF.

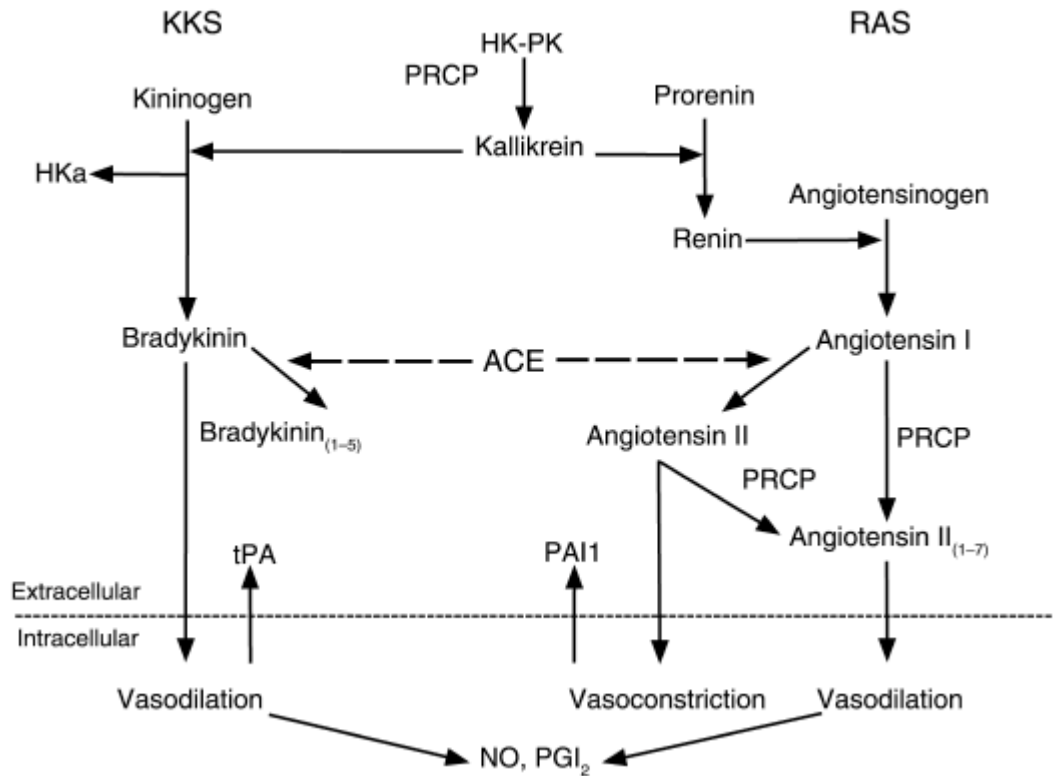


Fig 1 The interaction between the plasma KKS and RAS. Plasma kallikrein converts prorenin to renin, and renin has the ability to convert angiotensinogen to Ang I. ACE converts inactive Ang I to the vasoconstrictor Ang II. Ang II stimulates plasminogen activator inhibitor 1 (PAI1) release from endothelial cells. At the same time ACE degrades BK into BK(1–7) (not shown) or BK(1–5), a peptide with thrombin inhibitory activity. PRCP is the enzyme that degrades Ang II or Ang I to the vasodilating peptide, Ang II(1–7). Ang II(1–7) stimulates NO and PGI₂ formation, which potentiates the effects of BK. PRCP also has the ability to convert PK to kallikrein. Formed kallikrein digests kininogens to liberate BK, leaving a kinin-free kininogen (HKa) that has anti-proliferative and anti-angiogenic properties. Thus, PRCP, the same enzyme that degrades the vasoconstrictor Ang II, leads to the increased formation of the vasodilators BK Ang II(1–7). Finally, the resulting BK stimulates tPA, NO, and PGI₂ formation, thus counterbalancing the prothrombotic effect of Ang II (Schmaier, 2002).

Questions Addressed

Section I

The AT2 receptor is abundantly expressed in the adult uterus, and its levels depend on the menstrual cycle. Physiologically, the AT2 receptor seems to be involved in the control of menstrual cycle and reproduction. The expression of the AT2 receptor in malignant tumor cells of human uterus leiomyosarcoma SK-UT-1 cells may indicate a role of Ang II in a number of events associated with proliferation, differentiation or apoptosis.

First, it was intended to investigate whether the expression of the AT2 receptor in uterus tumor cells is regulated by growth factors.

The AT2 receptor has been demonstrated to inhibit growth in CEC (Stoll et al., 1995) and PC12W cells (Meffert et al., 1996). The question arises, whether an activation of the AT2 receptor promotes or inhibits proliferation of SK-UT-1 cells. If Ang II inhibits growth of these cells, does the peptide promote their differentiation? Is there reciprocal relationship between the degree of differentiation and the level of proliferation in these cells?

Ang II acting on the AT2 receptor can induce apoptosis in PC12W cells (Gallinat et al., 1999). Do the leiomyosarcoma cells also undergo apoptosis after their exposure to Ang II? Is these effect mediated by the AT2 receptor?

Section II

Former studies have demonstrated the presence of both angiotensin receptor subtypes in CEC. Generally, activation of the AT2 receptor induces growth inhibition. The AT2 receptor may exert its growth-modulatory- and differentiation-stimulatory actions on various levels including the modulation of the signaling of other receptors and the activity of growth- and transcription factors, as well as the remodeling of the extracellular matrix. Enhanced expression of *Zfhep* may represent one of the

mechanism by which the AT2 receptor promotes cell differentiation in various cells. In the second set of the experiments, It was investigated whether Ang II affects the gene expression of *Zfhep* in rat CEC. These cells express AT1 as well as AT2 receptors. Assuming that the *Zfhep* expression is regulated by Ang II in CEC, it would be important to demonstrate, which angiotensin receptor subtype mediates this effect.

Section III

Several studies have demonstrated that Ang II can increase cGMP levels. The mechanism involves activation of the B2 receptor by BK and NO formation. We have demonstrated previously, that the AT2 receptor is involved, since inhibition of this angiotensin receptor subtype completely abolished the Ang II-induced cGMP formation (Gohlke et al., 1998). Activation of the AT2 receptor induces differentiation in PC12W cells (Meffert et al., 1996) and this effect may involve the BK/NO cascade. Following questions are to be answered:

Does blockade of the B2 receptor or inhibition of NO synthases (NOS) attenuate the effect of Ang II on the differentiation of PC12W cells? Is the generation of cGMP a prerequisite of the differentiation in PC12W cells?

Can sodium nitroprusside, an exogenous NO-donor, induce differentiation in PC12W cells independently of AT2 receptor stimulation?

Providing that inhibition of NOS attenuates the Ang II-induced differentiation of PC12W cells, can an exogenous NO-donor reverse this effect?

Materials and Methods

Equipments

eukaryotic cell incubator: IG 150	Jouan, Unterhaching, Germany
bacterial incubator	Jouan, Unterhaching, Germany
bacterial incubator/Shaker: Innova 4000	New Brunswick Scientific, USA
eppendorf incubator: Thermomixer 5436	Eppendorf, Hamburg, Germany
water baths	GFL, Burgwedel, Germany
spectrophotometer: U-2000	Hitachi Ltd, Tokyo, Japan
benchtop centrifuge MR1822	Jouan, Unterhaching, Germany
centrifuge: Minifuge RF	Heraeus sepatech, Hannover ermany
electrophoresis apparatus: Horizon 11-14	Gibco BRL, Eggenstein, Germany
video imaging documentation system	Intas, Germany
hybridization oven	Biometra, Göttingen, Germany
electrophoresis apparatus: Vertical 15-17	Gibco BRL, Eggenstein, Germany
β -counter: Wallac 1409 liquid	
scintillation counter	Berthold GmbH,Wilddbad,Germany
γ -counter: Wallac 1470 Wizard	Berthold GmbH,Wilddbad,Germany
sonicator: Sonoplus GM70	Bandelin electronics, Berlin, Germany

Angiotensin receptor ligands

PD123177, PD123199, losartan were kindly gifts from respectively:

Dr. David Taylor at Parke Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor MI, USA.

Dr. Marc de Gasparo at Novartis, Pharmaceutical Research Division, Basel, Switzerland.

Dr. Ronald Smith at Dupont Merck Pharmaceutical Company, Wilmington DE, USA.

Angiotensin II

Sigma, Deisenhofen, Germany

Growth factors

bFGF, human recombinant growth factor

Hofmann La Roche Mannheim
Biochemica, Mannheim,
Germany

NGF

Sigma, Deisenhofen, Germany

Salts, acids, organic solutions:

NaCl, MgCl₂ EDTA, HCl, acetic acid, ethanol, etc

Merck, Darmstadt, or Sigma
Deisenhofen, Germany

General cell culture conditions

Cell lines:

SK-UT-1 cells (ATCC HTB 114) were kindly provided by Dr. Hendrik Ungefroren, Kiel Germany.

PC12W cells, were kindly provide by Dr. Serge Bottari, Grenoble, France.

The coronary endothelial cells (CEC) were isolated by from male Wistar rats (Stoll et al., 1995).

Reagents

Dulbecco's modified essential medium (DMEM) without sodium pyruvate, with 4500 mg/l glucose

GibcoBRL, Eggenstein, Germany

Dulbecco's modified. essential medium

Gibco BRL, Eggenstein, Germany

(DMEM) with 1000 mg/L Glucose, with sodium pyruvate, with pyridoxine

without phenol color

Nutrient mixture F-12 (HAM'-12)

Gibco BRL, Eggenstein, Germany

Fetal calf serum (FCS)

Biowhittaker Bioproducts

Walkersville MD, USA

Horse serum (HS)

Bioconcept, Umkirch, Germany

Trypsin (10 X)

Gibco BRL, Eggenstein, Germany

Trypsin/EDTA (10 X)	Gibco BRL, Eggenstein, Germany
Penicillin:streptomycin (10,000 IU/ml:10,000 µg/ml)	Gibco BRL, Eggenstein, Germany
PBS: 140 mM NaCl; 2,8 mM KCl; 8.1 mM Na ₂ HPO ₄ ; 1.5 mM KH ₂ PO ₄ ; 0.5 mM MgCl ₂ pH (7.4)	Seromed, Berlin, Germany
L-Glutamin (100 X)	Gibco BRL, Eggenstein, Germany

Culture dishes:

10 cm dishes or 6 well, 24 well plates and 6 well plates	Nunc, Wiesbaden, Germany
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Protocols:

SK-UT-1 cells were cultured under combination with DMEM (without phenol color) and Ham'-12 as 1:1, supplemented 1% penicillin:streptomycin (100 IU/ml:100 µg/ml) and 10 % iFCS, 2 mM of L-Glutamin. The cell line was passed using 1.5 X trypsin/EDTA.

PC12W cells were kept under DMEM (without sodium pyruvate) supplemented with 10% iHS, 5% iFCS and 1% penicillin:streptomycin (100 IU/ml:100 µg/ml). This cell line was passed using 1 X passage/EDTA. PC12W cells were used from passage 14 to 17.

Coronary endothelial cells (CEC) were isolated from male Wistar rats and cultured in DMEM with 10% iFCS and 1% penicillin:streptomycin (100 IU/ml:100 µg/ml) and passed with 1 X trypsin/EDTA.

All cells were usually grown as a monolayer in appropriate dishes at 37°C in a humidified atmosphere of air/CO₂ (19:1). To achieve quiescent cells, SK-UT-1, PC12W cells and CEC were serum-deprived for 48 hrs prior to the experiments.

Receptor radioligand binding assay

Expression of receptors on whole cells can be detected with radio-labeled ligands. Competition for the binding site between the radioactive labeled Ang II and an excess

of the AT1 or the AT2 specific ligands allowed the detection of angiotensin receptor subtypes on the cell surface.

Reagents:

Ligands: see section “angiotensin receptor ligands“

[¹²⁵ I] Tyr ⁴ -ANG II (agonist)	NEN life Science Products
Specific activity of 2200 Ci/mmol	Boston MA, USA

Protocol:

Cells were grown as a monolayer in 24 well dishes (500 µl medium/well). [¹²⁵I] Tyr⁴-ANG II (40 pmol/single well) was usually added to the medium (500 µl/well) for 2 hrs, at 37°C. The cells were washed twice with ice-cold PBS and solubilized in 1 N NaOH for 20 min at 37°C. The solubilized material was then counted for γ-radioactivity. Non-specific binding was performed in parallel cultures, incubated with [¹²⁵I] Tyr⁴-ANG II in combination with 1 µM Ang II and was usually less than 2% of total binding. To determine the receptor binding specificity, cells were incubated with [¹²⁵I] Tyr⁴-ANG II in combination with specific angiotensin receptor antagonists (1 µM each).

Thymidine incorporation assay

Cells were cultured in the presence of [methyl,1,2-³H]-thymidine to measure the capability of Ang II to affect growth activity through the activation of the AT1 and/or AT2 receptor. The amount of labeled thymidine incorporated into the DNA of the cells is a parameter of cell growth activity.

Reagents:

[methyl,1,2- ³ H]-thymidine, specific activity: 90-120 Ci/mmol	NEN life Science Products Boston MA, USA
Liquid scintillation cocktail: Ready Protein+	Beckman, Frankfurt, Germany
Fixative solution: 10% trichloroacetic acid solution	Merck, Darmstadt, Germany

Protocol:

SK-UT-1 cells were plated in 24 well plates at concentration of 5×10^4 /ml and left to grow for 24 hrs. Cells were serum deprived for 48 hrs prior to the thymidine assay. Then, Ang II alone or Ang II together with the AT1 or the AT2 antagonist was added with [methyl,1,2-³H]-thymidine (1 μ Ci/well) to the appropriate medium for 24 hrs. On the following day, the radioactive media were removed and the cells were washed with cold PBS (0.5 ml/well). The cells were fixed with 10% trichloroacetic acid solution (0.5 ml/well) overnight in an incubator at 37°C. Subsequently, the fixative solution was removed, followed by a wash with cold PBS. The cells were lysed in NaOH (0.1 N 400 μ l/well) for 1 hr at room temperature by shaking at 250 rpm/min. Finally, 200 μ l of the lysate were transferred to scintillation vials containing 4 ml of liquid scintillation cocktail solution. The radioactivity was counted in a β -counter for 5 min.

Cell counting procedure

To measure the growth activity, cells were counted using a hemocytometer under a conventional light microscope.

Reagents:

Trypan blue dye	Sigma, Deisenhofen, Germany
Trypsin/EDTA (10X)	Gibco BRL, Eggenstein, Germany
Phosphate-buffered saline (PBS)	Seromed, Berlin, Germany

Protocol:

SK-UT-1 cells were plated at concentration of 5×10^4 cells/ml in 24 well dishes for 24 hrs and then serum deprived for 48 hrs to obtain quiescent cells. On the following day, cells were incubated with Ang II in the presence or absence of the selective AT1 and AT2 receptor antagonist, respectively, for 16-24 hrs. After wash with PBS, the cells were incubated with 100 μ l of 1.5 X trypsin/EDTA solution for 3-4 min, suspended in 900 μ l of PBS and centrifuged. The pellets were re-suspended in PBS

solution and 0.4%(w/v) trypan blue was added (viable cells do not take up the dye). A fraction of the cell mixture was pipetted onto the hemocytometer and the cells were counted under the light microscope.

RNA isolation procedure

RNA was isolated from cells and depending on the experimental protocol, prepared for Northern Blot or RT-PCR. This protocol describes the RNA isolation method from whole cells using GTC buffer according to the protocol by Chomczynski and Sacchi (Chomczynski & Sacchi, 1987) or Trizol-reagent according to the manufacturers' recommendations.

Reagents:

Guanidine Thiocyanate	Sigma, Deisenhofen, Germany
N-Lauroylsarcosine	Sigma, Deisenhofen, Germany
β -mercaptoethanol	Sigma, Deisenhofen, Germany
Sodium acetate	Sigma, Deisenhofen, Germany
Phenol (water saturated) capped with layer buffer, pH,4.2)	ICN Biomedicals, Ohio, U.S.A
Chloroform	Merck, Darmstadt, Germany
Isoamylalcohol	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Diethyl pyrocaronate (DEPC)	Sigma, Deisenhofen, Germany
N-Lauroylsarcosine	Sigma, Deisenhofen, Germany
Trizol-reagent.	Gibco BRL, Eggenstein, Germany

Protocol:

The cells were cultured in 10 cm dishes until they reached different states of confluence (according to experimental requirements). The RNA was isolated using either the Trizol-reagent method or the GTC-buffer method.

Trizol-reagent method:

2 ml of Trizol-reagent were added to each dish and incubated for 10 min at room temperature. The lysed cells were transferred into 1.5 ml Eppendorf tubes. 200 μ l chloroform were added per 1 ml of Trizol-cell solution. The Eppendorf tubes were inverted several times and centrifuged at 13,000 rpm for 15 min, which resulted in the separation of the aqueous and organic phases. The upper aqueous phase containing the RNA was transferred into separate Eppendorf tubes. 500 μ l of isopropanol were added and left for 15 min at room temperature. The precipitated RNA was collected by centrifugation at 13,000 rpm for 15 min. The RNA pellet was washed by adding 1 ml of 75% ethanol centrifuged at 7,500 g for 5 min. Ethanol was removed and the pellet was left to dry for no longer than 10 min at room temperature.

GTC-buffer method

2 ml of GTC buffer (4 M guanidine thiocyanate, 5 g/l w/v, N-Lauroylsarcosine, 0.0072% v/v, β -mercaptoethanol) were added to each dish. Repeated re-pipetting lysed the cells, sheared the DNA and reduced the viscosity. The cell lysate was transferred into separate 2 ml Eppendorf tubes and incubated in ice for 10 min. Sodium acetate solution (2 M, pH 4.0, 1/10 of the lysate volume) was added and the tubes were inverted several times. The same volume of water-saturated phenol solution was added and the tubes were mixed again. Then, chloroform/isoamylalcohol (49:1, 1/10 volume of the original lysate volume) was added and mixed by inverting the tubes several times. After incubation for 15 min in ice bath, the solutions were separated by centrifugation (12,000 g for 20 min at 4°C). Upper phase containing the RNA was transferred into a 2 ml Eppendorf tube. The RNA was precipitated by adding of isopropanol (the same volume) and keeping the tubes in a refrigerator at -20°C for 30 min. The RNA was collected by centrifugation (12,000 g for 20 min at 4°C). The RNA pellets were washed with 1 ml 100% ethanol followed by centrifugation (12,000 g, 10 min, at 4°C). Ethanol was removed and discarded. 1 ml of 75% ethanol was added and the tubes were centrifuged again (7,500 g, 10 min, 4°C). Ethanol was removed and the pellet was left to dry for no longer than 10 min at room temperature.

The following steps are the same for both methods.

RNase free water was added (50 - 100 µl). An aliquot of 2 µl was taken for the determination of the RNA quantity and quality using a spectrophotometer. For this purpose, 2 µl of RNA solution were diluted with 198 µl of pure water and transferred to a crystalline cuvette to measure the RNA content.

An optical density (OD)_{260nm} value of 1 represents 40 µg of RNA/ml and an OD₂₆₀:OD₂₈₀ ratio of 1.6 to 2.0 indicates a high quality grade of the RNA. The RNA was stored at -80°C until it was used for Northern Blot or reverse transcription (see appropriate sections).

Northern Blot and hybridization

Northern Blotting and hybridization are used to determine the quality, quantity and size of the isolated RNA. This protocol describes the preparation of a RNA gel, the transfer of the RNA from the gel to a nylon membrane by a capillary blotting procedure and subsequently the hybridization of a radio-labeled cDNA probe to its corresponding RNA sequence fixed on the membrane.

Reagents:

Agarose	Promega, Mannheim, Germany
Hybond-N; nylon membrane:	Amersham International plc Buckinghamshire, UK
10 mg/ml ethidium bromide	Sigma, Deisenhofen, Germany
2.5 mg/ml bromophenol blue	Sigma, Deisenhofen, Germany
Rapid-Hyb buffer	Amersham International plc Buckinghamshire, UK
Random prime labelling system (rediprime TM II)	Amersham International plc Buckinghamshire, UK
Scientific imaging film, Biomax MS	Eastman Kodak Company, Rochester NY, USA
3 MM Whatman chromatography paper	Biometra GmbH, Göttingen, Germany
37% formaldehyde	Sigma, Deisenhofen, Germany
10 X MOPS running buffer (pH 7.0):	
200 mM 3-(N-morpholino) propane-sulphonic acid, 80 mM sodium acetate, 10 mM EDTA (final pH 7.0)	

RNA loading buffer :

10 µl formamide, 4 µl formaldehyde, 3 µl 10 X MOPS buffer

20 X SSPE:

3.6 M NaCl, 0.2 M NaH₂PO₄ (pH 7.4), 0.2 M EDTA (final pH 7.4)

Orange dye : Sigma, Deisenhofen, Germany

0.01 M EDTA (pH 7.0), 0.05 mM Ficoll 400, 1% orange

Protocol:

A mixture of 73.3 ml distilled water and 1.0 g agarose was boiled in a microwave. Afterwards, the gel solution was cooled to 65°C and 16.7 ml of 37% formaldehyde and 10 ml of 10 X MOPS were added. The solution was thoroughly swirled and poured into a gel tray. A comb was placed into the gel and left to solidify for at least 30 min at room temperature and then 1 hr at 4°C. After the polymerization, the gel was placed into the electrophoresis apparatus submerged in 1 X MOPS electrophoresis buffer. The comb was then carefully removed.

Meanwhile, 10-20 µg of RNA samples had been dried previously in a speedvac centrifuge and re-suspended in 10 µl of DEPC water. 17 µl of RNA loading buffer were pipetted onto each RNA sample, mixed and briefly centrifuged. The loading buffer/RNA solution mixture was incubated at 68°C for 5 min and chilled in ice for 5 min. Prior to loading, 4 µl of orange (1/10) dye was added to the mixture. After loading, the gel was run at 18 mA for 17.5 hrs (or 60 V for 6 hrs).

On the following day, the gel was stained in 0.5 µg/ml ethidium bromide 1 X MOPS buffer for 30 min and de-stained with 1 X MOPS for 30 min. The gel was washed with 3 different buffers for 30 min on a shaker at room temperature. The following buffers, all prepared in DEPC water, were used: NaOH-NaCl (50 mM NaOH, 10 mM NaCl), 0.1 M Tris-HCl (pH 7.4) and in 20 X SSPE. After taken a photograph of the gel, the gel was inverted and placed upon a wick covered with one piece of 3MM Whatman chromatography paper, which was set up in a tray containing 20 X SSPE solution. A single nylon membrane sheet, cut to the exact gel size and pre-wetted first in DEPC water and then in 20 X SSPE for at least 5 min, was placed upon the gel and oriented by one corner cutting. 3 - 5 pieces of 3 MM Whatman chromatography papers of the same size were placed on the top followed by a stack of paper towels, all cut to the exact gel size. A 500 g weight was put on the top of the

paper towels. The transfer of RNA from the gel to the nylon membrane was completed after 8-18 hrs. The contact between the sheets and the wick was carefully avoided by using a slim slip of Parafilm to prevent a "short-circuit" which would have led to inefficient transfer of the RNA from the gel to the nylon membrane.

On the following day, the nylon membrane blot was removed from the gel and prepared for hybridization. The blot was first air dried on a sheet of dry Whatman paper. Upon verification of the RNA quality and quantity by holding the membrane against ultraviolet light, the blot was prepared for hybridization. Typical for the eukaryotic RNA is the prominent presence of 28S- and 18S- RNA. Both types are clearly visible under UV light as two prominent bands. The presence of a smear rather than two distinct bands indicates the loss or total degradation of the RNA population. The density of the bands on the blot indicates the same amount of sample loaded. The blot was then packed with two pieces of 3 MM Whatman paper, and dried for 2 hrs at 80°C in an oven to fix the RNA, and stored at -80°C till hybridization.

Before the hybridization, the blot was wetted in 200 ml of sterilized distilled water, washed in 200 ml of 5 X SSPE solution for 5 min and then placed into a glass hybridization tube containing 20 ml of pre-warmed (65°C) Rapid-Hyb solution. The tube was placed into a hybridization oven, allowing it to rotate at 65°C for at least 30 min. During this time, a radioactive labeled cDNA probe was prepared (see section: synthesis of radiolabeled cDNA probe). After the pre-hybridization period, the Rapid-Hyb solution was removed from the tube and 20 ml of fresh Rapid-Hyb solution, containing the radioactive probe, were added. The tube was again placed into the hybridization oven and left rotating at 65°C for 1 hr.

After the hybridization period, the blot was subjected to a series of washes of increasing stringency, to remove non-specifically bound radioactive probe. The washing procedure was usually as follows: twice at 50°C for 5 min in 2 X SSPE, 0.1% SDS, once at 50°C for 20 min in 2 X SSPE, 0.1% SDS, twice at 65°C for 20 min in 0.3 X SSPE, 0.1% SDS. The blot was wrapped in transparent cling membrane, laid into a cassette a covered with a light sensitive film. The film was exposed at -80°C for 1 day, 3 days and/or 7 days.

Synthesis of radio-labeled cDNA probes

Several protocols exist to synthesize the radio-labeled cDNA probe. In the random priming method, the short DNA fragment to be labeled is first denatured and then mixed with oligonucleotides of random sequence in the presence of labeled nucleotide(s) and a DNA polymerase. These "random oligos" anneal to complementary random sites on the DNA and serve as primers for DNA synthesis during which the label is incorporated.

Reagents:

Random prime labeling system	Amersham International plc, Buckinghamshire, UK
Rediprime™II	Amersham International plc, Buckinghamshire, UK
[α - ³² P]dCTP specific activity: 3000 Ci/mmol	Amersham International plc, Buckinghamshire, UK
Rapid-Hyb buffer	Amersham International plc Buckinghamshire, UK

TE-buffer (pH 8.0):

10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)

Protocol:

The Random prime labeling system was used to generate radioactive labeled DNA probes and the procedure was performed according to the manufacturers' recommendations. 2 μ l of target DNA (see the section: preparation and transformation of competent cells) prepared as described elsewhere (see the section: plasmid DNA preparation, DNA precipitation, gel electrophoresis and gel isolation) were added into 43 μ l of TE buffer in a 1.5 ml Eppendorf tube and boiled for 5 min in a water bath and left to cool in ice for at least 10 min. The denatured cDNA solution was transferred into the tube containing labeling reaction (oligonucleotides of random sequence in the presence of labeled nucleotide(s) and a DNA polymerase). 5 μ l of [α -³²P] was added to the cDNA mixture and pipetted up

and down and the solution was briefly mixed. The reaction mixture was incubated at 37°C for 10 min and then 5 µl of 0.2 M EDTA were added. The mixture was boiled for 5 min then chilled in ice to terminate the reaction. The non-bound radioactivity [α -³²P] was separated from the labeled DNA by chromatography. The labeled cDNA probe was added into 20 ml fresh Rapid-Hyb buffer used for blotting (see section: Northern Blot and hybridization).

Target DNA preparation and transfection into competent cells

This protocol describes the preparation of target DNA for Northern Blot and transfection the DNA fragment into competent cells by original TA cloning kit. The PCR product inserted into the pCR[®] 2.1 plasmid vector and transformed into One Shot[™] cells by heat shock which allows these cells to accept foreign DNA. Individual recombinant plasmids were analyzed by restriction mapping for orientation. The correct recombinant plasmid was then purified for further experiments.

Reagents:

Original TA Cloning[®] Kit

(containing: 10 X Ligation buffer, T4 DNA Ligase, SOC medium, INV α F' one shot[®] competent cells)

Invitrogen, Groningen,
The Netherlands

Luria-Bertani (LB) broth

Sigma, Deisenhofen, Germany

LB agar (Lennox L agar)

Sigma, Deisenhofen, Germany

Protocol:

All medium and buffers used in this protocol were either autoclaved or filter sterilized. The procedure was performed according to manufacturers' recommendations.

Ten µl of fresh PCR product sample (usually 35 cycles, see section of PCR) sample were purified on 1-2 % of agarose gel electrophoresis, separated using a spin column (see the section, DNA gel electrophoresis and gel isolation). Ten µl of ligation reaction solution containing the PCR product, were prepared as follows: Five

µl of sterile water, 1 µl of 10 X ligation buffer, 2 µl of pCR[®] vector (25 ng/µl), 1 µl of fresh PCR product and 1 µl of T4 DNA ligase. The reaction solution was incubated at 14°C overnight for transformation.

Vials containing 50 µl of frozen INVαF' competent cells (One Shot[®] competent cells) were thawed on ice. 2 µl of 0.5 M β-mercaptoethanol were pipetted into each vial and gently mixed. Then, 2 µl of ligation reaction solution were directly pipetted into the vials containing INVαF' competent cells and gently mixed. After incubation on ice for 30 min, the cells were shock-heated for 30 sec at exactly 42°C and cooled on ice for 2 min. 250 µl of SOC medium (at room temperature) were added to each vial, incubated at 37°C for exactly 1 hr at in an incubator and then cooled on ice. 50 or 100 µl of INVαF' cells were spread onto LB agar plates containing 50 µg/ml of ampicillin. The plates with the spread cells were inverted and incubated at 37°C in an incubator for at least 18 hrs and then at 4°C for additional 2-3 hrs. 5-10 pieces of colonies from different areas of the plate were chosen and analyzed for the orientation of the insert in the plasmid.

Minipreps of plasmid DNA

Plasmids are able to replicate in bacterial cultures grown in the presence of the selective antibiotic. This protocol describes the plasmid DNA recovery procedure. The procedure was performed according to the manufacturers' recommendations.

Reagents:

Qiagen plasmid combi kit

Qiagen GmGH, Hilden, Germany

LB broth

Sigma, Deisenhofen, Germany

Protocol:

A single bacterial colony from an overnight culture grown on an agar plate was transferred to 5 ml LB medium containing ampicillin (50 µg/ml). The culture was incubated with shaking (250 rpm) at 37°C overnight. On the following day, the bacteria were collected by a 5 min spin at 3,000 rpm. The bacteria pellet was resuspended in 300 µl of buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100

μg/ml RNase A). 300 μl of buffer P2 (200 mM NaOH, 1% SDS) were added and the mixture was gently swirled for 5 min to lyse the cells. Additional 300 μl of buffer P3 (3.0 M potassium acetate, pH 5.5) were added which caused immediately precipitation of proteins. The mixture was rapidly but gently mixed and immediately centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was applied to the tips, which had been equilibrated with 1 ml of buffer QBT (0.75 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100) and emptied by gravity flow. The tips were 4 times washed with 1 ml of buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol). The DNA in the tip was eluted by 0.8 ml buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol) and precipitated with 0.7 volumes of isopropanol. After centrifugation at 10,000 rpm for 30 min and wash with 1 ml of 70% ethanol, the DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). For spectrophotometrical analysis, 2 μl of DNA stock solution were pipetted into 198 μl of water and transferred to a crystalline cuvette. An OD_{260 nm} value of 1 represents to 50 μg of DNA/ml and OD_{260nm}:OD_{280nm} ratio of 1.6 to 2.0 indicates a high quality grade of the DNA. The DNA was stored in water at -20°C .

DNA restriction procedure

DNA fragments isolated from plasmid were cut with appropriate enzymes and isolated by DNA electrophoresis. This protocol describes the restriction used through the research project.

Reagents:

DNA restriction enzyme	Gibco RBL, Eggenstein, Germany
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10 X restriction endonuclease buffer:

100 mM Tris-HCl (pH 7.5), 100 mM MgCl ₂ , 10 mM DTT, 1 mg/ml BSA, 0, 0.5, 1.0, 1.5 M NaCl	Gibco RBL, Eggenstein, Germany
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(NaCl concentration vary depending on the enzyme used. These four different concentrations cover most commercially available restriction enzyme).

Protocol:

DNA, usually 2 µg, was cut in a reaction volume of 25 µl at 37°C for 1 hr or overnight in the presence of 1 X restriction endonuclease buffer. The reaction was terminated by a phenol:chloroform extraction (1:1) and ethanol precipitation. The fragments of DNA was checked with DNA gel electrophoresis (see section: DNA gel electrophoresis and gel isolation) and the appropriate DNA was isolated from the gel and used for Northern Blot.

DNA precipitation, gel electrophoresis and gel isolation

DNA fragments were run on an agarose gel to analyze their quality and size. Additionally, once DNA fragments were separated on a gel, it was possible to isolate them for subsequent radio-labeling or cloning procedures. This protocol describes the preparation of a DNA gel and electrophoresis, and the isolation procedure of a single DNA from a gel.

Reagents:

Agarose	Promega, Mannheim, Germany
gel extraction kit	Qiagen GmbH, Hilden, Germany
10 mg/ml ethidium bromide	Sigma, Deisenhofen, Germany

TBE buffer (1 liter, 10 X stock solution):

108 g Tris base, 55 g boric acid, 40 ml of 0.5 M EDTA (pH 8.0)

Loading buffer (10 X):

50% (v/v) glycerol, 0.1 M EDTA (pH 8.0), 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol

Protocol:

To 100 ml of 1 X TBE, 1.0 g of agarose was added and brought to boil in a microwave. The boiling solution was thoroughly swirled and poured into a prepared gel tray. A comb was inserted. The gel was left to polymerize and kept at 4°C at least 1 hr before being used. The gel was placed into the electrophoresis apparatus

and submerged in 1 X TBE electrophoresis buffer after which the comb was carefully removed.

Prior to a gel run, the DNA dissolved in ultra-pure water was mixed with 10 X loading buffer. The mixture was loaded into the gel slot and subjected to an electrical field of 90 V for at least 1 hr. The gel was then stained with ethidium bromide (5 µg/ml) for 20 min. The DNA fragments were visualized under ultraviolet light and photographed using a video documentation system.

DNA fragments were isolated from the gel using a commercially available kit. The procedure was performed according to the manufacturers' recommendations. Briefly, the band containing the DNA fragment of interest was cut from the gel under the UV and placed in an Eppendorf tube. Solubilization buffer was added and the mixture was incubated at 50°C for 10 min. The dissolved gel mixture was transferred to a spin column designed to trap DNA on a glass fibre filter. The filter was washed twice by adding 700 µl of provided buffer followed by centrifugation step of 13,000 rpm for 1 min. After the washing procedure, 50 µl of ultra-pure water were added to the glass fibre filter which was left at room temperature for 5 min to elute the DNA. The column was spun at 13,000 rpm for 1 min and the DNA was recovered in a collection tube. The purified DNA could be used immediately for radio-labeling or cloning procedures.

Reverse transcription reaction

The enzyme reverse transcriptase was used to generate DNA molecules from RNA molecules. Like the DNA polymerases, this enzyme can build a complementary nucleic acid strand on a template by adding nucleotides to a hybridized short DNA fragment or primer.

Reagents:

Total RNA isolated from whole cells (for procedure see section: RNA isolation)

Superscript reverse transcription kit Gibco BRL, Eggenstein, Germany

10 X reverse transcription buffer:

200 mM Tris-HCl (pH 8.4), 500 mM KCl

Protocol:

To 5 µg of total RNA, 1 µl of oligo(dT) primers (0.5 µg/µl) and DEPC treated water were added up to a volume of 12 µl. The mixture was heated to 70°C for 10 min and cooled on ice for at least 1 min. Meanwhile, a stock solution of 2 µl 10 X reverse transcription buffer, 2 µl of MgCl₂ (25 mM), 1 µl of nucleotides (10 mM each) and 2 µl DTT (0.1 M) was prepared. The prepared mixture was pipetted into the oligo(dT)/RNA solution which was incubated at 42°C for 5 min. Following the brief incubation period, 200 units of Superscriptase for each sample were added and the tubes were incubated at 42°C for additional 50 min. The reaction was terminated by heating the tubes to 70°C for 15 min and by subsequent cooling on ice. To destroy the RNA template, 2 units of RNase H were added to the reaction mixture, which was then incubated at 37°C for 20 min. Finally, the cDNA mixture was stored at -20°C until use in the PCR.

Polymerase chain reaction (PCR)

The PCR is a rapid procedure for an *in vitro* enzymatic amplification of a specific segment of DNA. A typical PCR mixture contains the target DNA, the oligodeoxynucleotides (short DNA fragments encoding for the extreme ends of the target DNA) in much higher concentration than the target DNA, free nucleotides and a heat stable DNA polymerase with suitable buffer components. The mixture is heated to 95°C for 1 min to denature the target DNA followed by a cooling step to 55-65°C for 1 min to allow the oligodeoxynucleotides to hybridize into their complete DNA sequences. The mixture is brought to 72°C, which is the optimum temperature for the *Taq* polymerase to extend the hybridized oligodeoxynucleotides. When the synthesis is complete the mixture is heated further to 95°C to melt the synthesized DNA duplexes. When the temperature is lowered, another round of synthesis can take place because an excess of oligodeoxynucleotides is still present. The cycle of melting, hybridization and synthesis is repeated many times. At each round the number of copies of the sequence of interest is doubled and therefore the number of copies increases exponentially. A single target DNA molecule provides 2ⁿ copies, with n being the number of cycles.

Reagents

<i>Taq</i> polymerase	Gibco BRL, Eggenstein, Germany
Nucleotide(s)	Roche, Mannheim, Germany
human AT1 receptor primers	Pharmacia Biotech, Germany
human AT2 receptor primers	Pharmacia Biotech, Germany
human calponin primers	Pharmacia Biotech, Germany
human SM22 primers	Pharmacia Biotech, Germany
rat iNOS primers	Pharmacia Biotech, Germany
rat eNOS primers	Pharmacia Biotech, Germany
rat nNOS primers	Pharmacia Biotech, Germany
rat AT1 receptor primers	Pharmacia Biotech, Germany
rat AT2 receptor primers	Pharmacia Biotech, Germany

Protocol

The following components were pipetted into an Eppendorf tube: 5 µl 10 X PCR buffer, 2 µl of MgCl₂ (50 mM), 4 µl of nucleotides (final concentration: 0.125 µM each), 1 µl of primers (10 mM each), 0.5 µl *Taq* (5 µg/µl), 1 µl of cDNA stock solution and ultra-pure water up to 50 µl.

The mixture was placed in a thermo cycler and heated to 95°C for 5 min and submitted 35-times to 95°C for 30 sec, 56°C (temperature profiles were adjusted depending on the specific primer sequences) for 30 sec and 72°C for 1 min. Following the cycle procedure, the mixture was incubated at 72°C for 7 min and then kept at 4°C until use.

Cytoplasm proteins isolation and measurement

This protocol describes the isolation procedure of total proteins from cell plasma. Various protein enzyme inhibitors were used in the protocol. The total proteins were measured by the protein assay kit according to the manufacturers' recommendations.

Reagents:

Aprotinin	Roche, Mannheim, Germany
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Leupeptin	Roche, Mannheim, Germany
TPCK	Roche, Mannheim, Germany
Lysis buffer:	
50 mM Tris-HCl (pH 8.0); 0.25 M NaCl; 0.5% NP-40; 1 mM PMSF; 1 µg/ml aprotinin; 1 µg/ml leupeptin; 20 µg/ml TPCK	
Bio-Rad protein assay	BIO-RAD, Copenhagen, Denmark

Protocol:

To obtain cell pellets, the cells were harvested with 800 µl of PBS once and centrifuged at 3,000 rpm for 5 min. The cell pellets were re-suspended with 200 µl of lysis buffer on ice for 30 min. After centrifugation at 13,000 rpm for 20 min the supernatant containing crude proteins was transferred into another Eppendorf tube and stored at -80°C till further analysis.

The Bio-Rad reagent was diluted with distilled water as 1:5. The BSA stock solution was diluted into 3 different working solutions (0.1; 0.2; 0.4 µg/µl) with distilled water. The sample proteins were diluted 1:50 with distilled water. 20 µl of diluted sample proteins or standard proteins were transferred into 1 ml of Bio-Rad reagent solution and incubated at room temperature for 10 min. OD_{595nm} of protein standards and samples were measured by spectrophotometer.

SDS Polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

The strongly anionic detergent sodium dodecyl sulfate (SDS) is used in combination with a reducing agent. The proteins are heated to denature before they are loaded on a gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is proportional to the molecular weight of the polypeptides and is independent of their sequence, SDS-polypeptide complexes migrate through polyacrylamide gels to the anode according to the size of the polypeptide. By using markers of known molecular weight, it is possible to estimate the molecular weight of the protein.

Reagents:

Ammonium persulfate (APS)(10%)	Sigma, Deisenhofen, Germany
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TEMED

Roche, Mannheim, Germany

Polyacrylamide solution (PAA)
(acrylamide:bisacrylamide 29%:1%)

Sigma, Deisenhofen, Germany

Separating gel solution:

10 % PAA, 375 mM Tris-HCl (pH 8.8); 0.1 % SDS

Stacking gel solution:

5 % PAA; 125 mM Tris-HCl (pH 6.8); 0.1 % SDS

5 X sample buffer:

60 mM Tris-HCl (pH 6.8), 2 % SDS, 14.4 mM 2-mercaptoethanol, 25 % glycerol, 0.1 % bromophenol blue.

Electrophoresis buffer:

275 mM Tris-HCl (pH 8.3); 200 mM glycine; 0.1 % SDS

Protocol:

Immediately before use, APS (final concentration is 0.1% v/v) and TEMEP (final concentration is 0.01%) were added to the gel solution. Upon preparation, the separating gel solution was pipetted between the assembled glass plate sandwich and covered with a layer of water and left to polymerize for 30 to 45 min. Subsequently, the water layer was removed and the prepared stacking gel solution was poured on the top of the polymerized separating gel. The comb was inserted and the gel was left to polymerize for additional 30 min. The prepared gel was assembled into the electrophoresis apparatus and submerged in electrophoresis buffer. The protein aliquots were diluted with distilled water to 20 μ l and 5 μ l of 5 X sample buffer were added. The mixture was incubated at 95°C for 10 min and then cooled on ice. The denatured samples were loaded onto the gel and subjected to electrical field of 150 V for 65 min. Upon completion of the gel run, proteins were transferred onto methanol activated nitrocellulose filter.

Western Blot

Virtually all western blots are carried out in two stages: an unlabeled antibody specific to the target protein is first incubated with the nitrocellulose filter containing the proteins in the presence of blocking solution. The filter is then washed and

incubated with a second antibody that is conjugated directly with horseradish peroxidase. After further washing, the antigen-antibody-antibody complexes on the nitrocellulose filter is located by an in situ enzymatic reaction resulting in the production of light, which can be detected using a light-sensitive film.

Reagents:

Monoclonal human anti-Calponin antibody	Sigma, Deisenhofen, Germany
Peroxidase-linked antimouse IgG	Amersham, Freiburg, Germany
ECL western blot detection reagents	Amersham, Freiburg, Germany

Protocol:

After incubation in blocking solution and briefly wash with 1 X TTBS, the membrane was immediately put into in a sealed box and incubated in a solution containing the primary antibody 5% of non-fat dried milk on a platform shaker at 4°C overnight. The membrane was washed three times with 1 X TTBS (15 min X 1 time and 5 min X 2 times) and incubated with the peroxidase-linked second antibody in a solution containing 2% of non-fat dried milk at room temperature for 1 hr. After the membrane had been transferred into an another tray, it was washed 5 times with 1 X TTBS (15 min X 1 time and 5 min X 4 times). All next steps were carried out in a dark room. Equal volumes of the detection solution 1 and the detection solution 2 were mixed together given a sufficient volume of the detection reagent to cover the membrane. The membrane was incubated in the detection reagent for 1 min at room temperature and then the excess of the detection reagent was drain off. The membrane was wrapped in transparent foil, fixed in the film cassette and exposed to autoradiography film for 3-5 min (in same cases for 10 min). The films were developed. The density of the bands correspond to the amount of proteins in the membrane.

Staining for apoptosis (TUNEL method)

Commercially available "In Situ Cell Death Detection Kit" allows the detection of cleaved DNA strands which are characteristic for apoptosis. DNA strand breaks can

be identified by labeling free 3'-OH termini with modified nucleotides incorporated by an enzymatic reaction. Free 3'-OH termini were elongated with fluorescein labeled nucleotides by terminal deoxynucleotidyl transferase (TDT) in a template-independent fashion. These nucleotides were in turn detected by an antibody conjugated with horseradish peroxidase. After a substrate reaction, apoptotic cells became stained and were detected by light microscopy.

Reagents:

In Situ Cell Death Detection Kit	Roche, Mannheim, Germany
TUNEL (TDT-mediated dUTP nick end labeling mixture: modified nucleotides, TDT, anti-fluorescein antibody)	
DNase I (RNase free)	Roche, Mannheim, Germany
TUNEL dilution Buffer	Roche, Mannheim, Germany
DAB Substrate (10 X DAB Metal Enhanced Substrate set, DAB Peroxide Buffer)	Roche, Mannheim, Germany
Paraformaldehyde	Sigma, Deisenhofen, Germany
H ₂ O ₂	Sigma, Deisenhofen, Germany
Permeabilization solution: 0.2 % Triton X-100 in 0.1 % sodium citrate	
Peroxidase dilute buffer (POD buffer)	Roche, Mannheim, Germany

Protocol:

The procedure was performed according to the manufacturers' recommendation. Briefly, the cells were plated in the 24 well plate for 72 hours. The treated cells were washed with PBS, dried by air and fixed using 4% of paraformaldehyde (pH 7.4) for 30 min at room temperature. The cells were then incubated in 0.3 % H₂O₂ in methanol for 30 min at room temperature and kept under permeabilisation solution for 15 min at room temperature after washing with PBS once. The cells serving as the positive control were pre-treated with DNase (100 µ/ml) at 37°C in a humidified chamber. All cells were then incubated in 50 % TUNEL mixture for 1 hr at 37°C in humidified chamber. Cells serving as the negative control were incubated with 50% TUNEL mixture without labeling enzyme (TDT). After rinsed with PBS three times,

the cells were incubated with converter horseradish peroxidase for an additional 1 hr at 37°C in a humidified chamber. The cells were then treated with 1 X DAB-substrate diluted with POD buffer and the reaction was allowed to run for 8 min at room temperature. Each incubation step was proceeded by three washing steps using PBS. The cells were photographed using light microscope.

Apoptotic DNA ladder

Cleavage of DNA in the cell is one of the hallmarks of apoptosis. To detect the apoptotic cells, cleaved DNA fragments can be analyzed using agarose gel after purification of DNA from cultured cells. Cells are lysed in binding buffer, the solution is transferred to a filter tube with glass fibre fleece and centrifuged. Nucleic acids bind specifically to the surface of glass fibres in the presence of chaotropic salts. Residual impurities are removed by a wash step and subsequently DNA is eluted with elution buffer.

Reagents:

Apoptotic DNA Ladder Kit	Roche, Mannheim, Germany
Agarose	Promega, Mannheim, Germany
Ethidium bromide	Sigma, Deisenhofen, Germany
Isoproponal	Sigma, Deisenhofen, Germany

Protocol:

SK-UT-1 cells were plated in 10 cm dishes and left to growth until 70% confluence. After the cells had been treated with various ligands and antagonists, they were harvested with 1.5 X trypsin/EDTA and re-suspended in 1 ml PBS solution. 200 µl of the cell-PBS solution (about 2×10^6 cells) were lysed in 200 µl lysis buffer during a 10 min incubation at room temperature. 100 µl of 100 % of isopropanol were used to precipitate the DNA and the resulting solution was transferred into filter tubes. After several washing steps, the DNA bound to the filter was eluted using elution buffer. Three µg of purified DNA from each sample was loaded to 1 % agarose gel (see section DNA precipitation, gel electrophoresis and gel isolation) and the gel was run

at 75 V for 4 hr. The apoptotic cells from the kit served as the positive control. DNA ladder was analyzed under UV and photographed for documentation.

Differentiation assay: Cell morphology

The outgrowth of axons and dendrites (together known as neurites) is a fundamental morphological event associated with neural development and differentiation. Therefore, the number of outgrowth neurites of certain length together with cell numbers represents an excellent marker for the cell differentiation. For the purpose of quantification, it seems to be useful to define at least three groups of neurites according to their lengths.

Reagents:

Collagen A (5 mg/ml)	Seromed, Berlin, Germany
NGF	Sigma, Deisenhofen, Germany
N _ω nitro-L-arginine methyl ester (L-NAME)	Sigma, Deisenhofen, Germany
8-bromoguanosine 3':5'-cyclic monophosphate (8-bromo-cGMP)	Sigma, Deisenhofen, Germany
Sodium nitroprusside (SNP)	Sigma, Deisenhofen, Germany

Protocol:

PC12 W cells in normal medium were planted at $1.4 \times 10^3/\text{cm}^2$ into the 10 cm dishes, which were pre-treated with collagen A : PBS (1:6) solution for 24 hrs. On the next day, the cells were serum deprived for 48 hrs to get quiescence. From day 4 to day 9 after planting, the cells were daily treated with desired tested substances (differentiation-inducer). The starve medium was changed daily. After 6 days of treatment, the cells were rinsed with PBS, and five randomly selected areas per one culture dish were photographed by phase-contrast microscopy. The obtained slides were projected on 4A papers and the cell numbers and neurite lengths, were marked on the papers for quantitative evaluation. Cell clusters (> 15 cells) were excluded. Outgrowth neurites were classified into three groups according to their length: small

neurites ($< 10\mu\text{m}$, $\cong < 5\mu\text{m}$), medium neurites ($10-20\mu\text{m}$, $\cong 5-10\mu\text{m}$) and large neurites ($> 20\mu\text{m}$, $\cong > 10\mu\text{m}$), and expressed as the numbers of neurite/100 cells

Data presentation

The results of cell counting procedure and thymidine assay in the proliferation study were representative for three to six separately performed experiments. For the cell counting, the data points presented per experiment correspond to an average \pm SEM of $n=6$ or 12 . For the thymidine assays, the data points presented per experiment correspond to an average \pm SEM of $n=4$. The data of PCR and western blot for differentiation as well as of TUNEL and DNA ladder for apoptosis were representative for at least three separately performed experiments. Photographs from PCR were quantified using the imaging software. The data in the differentiation study for PC12W cells were obtained from at least two independent experiments and all were presented as means \pm SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests.

APPENDIX to Material and Method

β - Actin Primer sequences:

Upper primer: 5' – ATG-GAT-GAT-GAT-ATC-GCC-GCG 3'

Lower primer: 5' – CAT-GAA-GCA-TTT-GCG-GTG-GAC-GAT-GGA-GGG-GCC 3'

Human AT1 receptor primer sequences:

Upper primer: 5' – GCA TTG ATC GAT ACC TGG CT – 3'

Lower primer: 5' – TTA CAT TAT CTG AGG GGC GG – 3'

Human AT2 receptor primer sequences:

Upper primer: 5' – GCT TGT GAA CAT CTC TGG CA – 3'

Lower primer: 5' – TTC ATT AAG GCA ATC CCA GC – 3'

Human calponin primer sequences:

Upper primer: 5' – AAA CAG GTG AAC GTG GGA G – 3'

Lower primer: 5' – TAG TTG TGT GCG TGG TGG TT – 3'

Human SM22 primer sequences:

Upper primer: 5' – ACA AGT CTT CAC TCC TCC CTG C-3'

Lower primer: 5' – TCA AAG AGG TCA ACA GTC TGG A-3'

Rat Zincfinger homoeodomain enhancer binding protein - primer sequences:

Upper primer: 5' – CAC ATT AAG TAC CGC CAT GAGA

Lower primer: 5' – GTT GTG CCA TCC TGA TCA ACTA

Rat iNOS primer sequences

Upper primer: 5' – GTT TTC TCC TTC CAC CGT GTA G – 3'

Lower primer: 5' – CAG GAG GCA GAG AAA GAC AGA T – 3'

Rat eNOS primer sequences

Upper primer: 5' – CCG CAC TTC TGT GCC TTT GCT C – 3'

Lower primer: 5' – GCT CGG GTG GAT TTG CTG CTC T – 3'

Rat nNOS primer sequences

Upper primer: 5' – AGA AGG AAC AGT CCC CTA CCT C – 3'

Lower primer: 5' – ACA GAT GTA GTT GAA CAT GCC G – 3'

Rat AT1 receptor primer sequences:

Upper primer: 5' – CAC GTG TCT CAG CAT CGA TCG – 3'

Lower primer: 5' – TGG CCA AGC CAG CCA TTA G – 3'

Rat AT2 receptor primer sequences:

Upper primer: 5' – AGT TCC CCT TGT TTG GTG TAT GG – 3'

Lower primer: 5' - CAT TAA GGC AAT CCC AGC AGA C – 3'

Results (section I)

Expression of Ang II receptor subtypes in human leiomyosarcoma cells

In the first study, the expression of AT1 and AT2 receptors in this cell line was determined by RT-PCR. The data from RT-PCR shows that both angiotensin receptors subtypes were expressed in these cells (Fig 1-1). In the presence of growth factors in the medium, the expression of the AT2 receptor was much weaker compared to that obtained in cells cultured in serum deprived medium for 48 hrs.

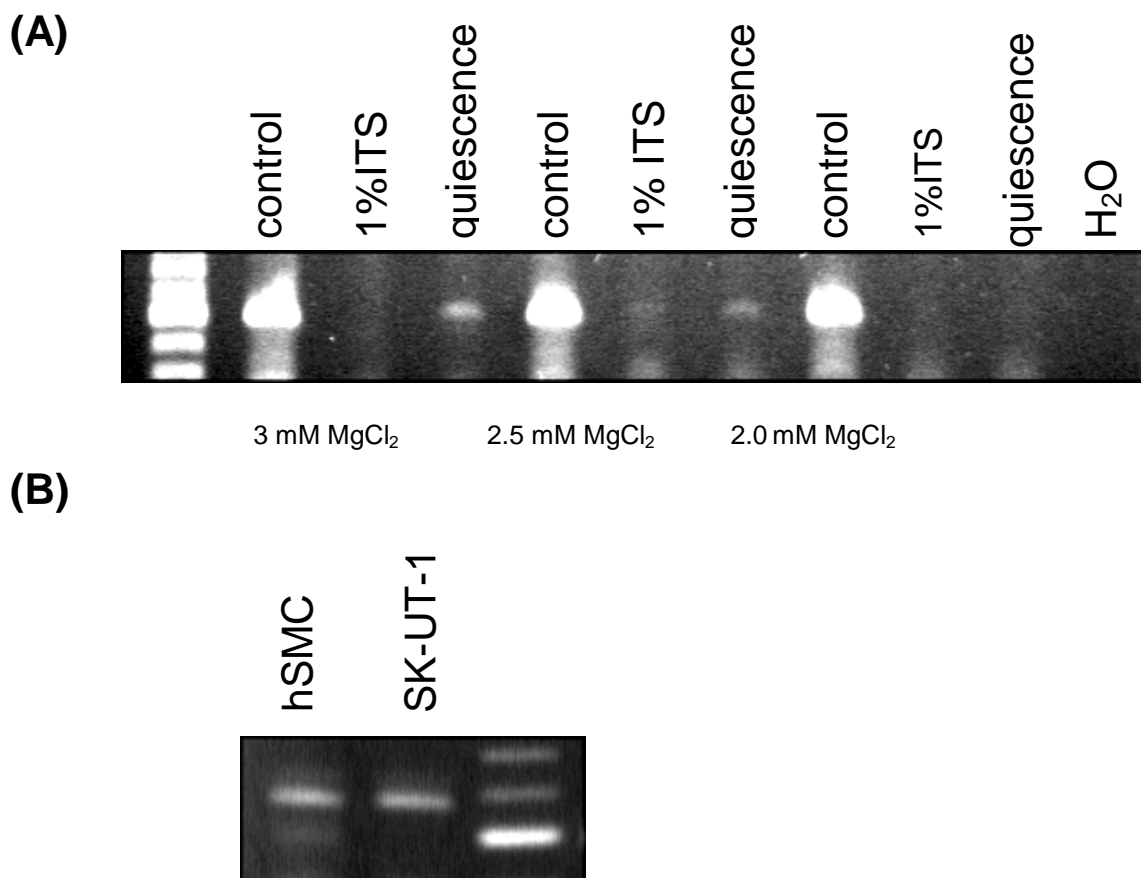


Fig 1-1. Expression of AT1 and AT2 receptors in SK-UT-1 cells. Total RNA was isolated from SK-UT-1 cells cultured either in normal medium or in starve medium. 5 μ g of total RNA were transcribed into cDNA and equal amounts of cDNAs were used for PCR with specific human AT1 and AT2 receptor primers, following 35 and 50 cycles respectively. Human umbilical SMC served as a positive control for the AT1 receptor PCR. Human genomic DNA was used as a positive control for the AT2 receptor PCR. 15 μ l of each PCR product were loaded on 1.2 % agarose gel and run at 90 V for 100 min. (A) PCR was performed using different concentration of MgCl₂ for the AT2 receptor. The AT2 receptor was identified by the product of 582 bp. (B) PCR for the AT1 receptor was identified by the product of 669 bp.

Effects of Ang II on the proliferation of quiescent SK-UT-1 cells

To investigate whether Ang II has any effect on the proliferation of leiomyosarcoma cells, SK-UT-1 cells (5×10^4 per well) were cultured in a 24 well-plate in normal medium containing 10% iFCS, 1% penicillin/streptomycin (100 IU/ml, 100 μ g/ml) and 2 mM L-glutamine. After 24 hrs, the medium was changed, and the cells were kept serum deprived for 48 hrs. For the cell counting, quiescent cells were stimulated with Ang II (10^{-7} M) in the presence or absence of angiotensin receptor antagonists, losartan (10^{-5} M) and PD123177 (10^{-6} M) for 16 to 20 hrs (overnight). Fig 1-2 shows that overnight treatment of quiescent SK-UT-1 cells with Ang II in the presence or absence of the AT1 or the AT2 receptor antagonists did not significantly change the number of cells. It can be concluded that in the absence of growth factors, stimulation of angiotensin receptors has no effect on the proliferation of leiomyosarcoma cells.

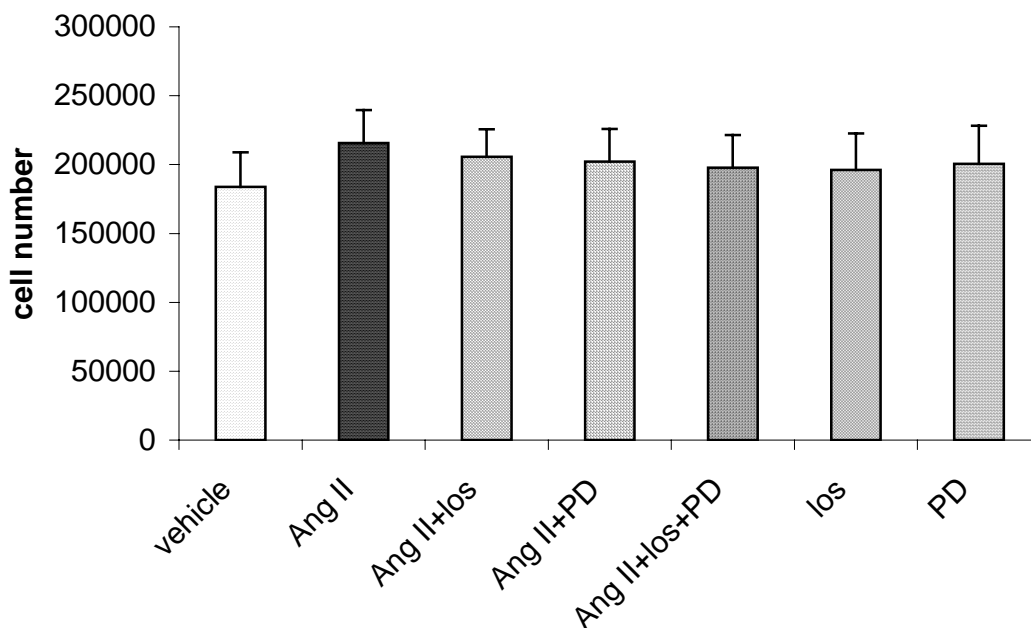


Fig 1-2. Effects of Ang II on the proliferation of quiescent SK-UT-1 cells. SK-UT-1 cells were planted in normal medium for 24 hrs to allow them to attach to the bottom of the dishes. Then the cells were serum deprived for 48 hrs to obtain quiescence. Ang II (10^{-7} M); Ang II (10^{-7} M) + losartan (los, 10^{-5} M); Ang II (10^{-7} M) + PD123177 (PD, 10^{-6} M) or Ang II (10^{-7} M) + losartan (10^{-5} M) + PD123177 (10^{-6} M) were added to the cells cultured in serum deprived medium. Cells were harvested by trypsin/EDTA solution and counted in a hemocytometer. The presented data are from a single experiment and representative of four independent experiments. Values represent means \pm SEM of $n=12$.

Effect of Ang II on the proliferation of SK-UT-1 cells in the presence of growth factors

It has been suggested that the effect of Ang II on the cell proliferation may require growth factors. Cell counting and ^3H thymidine incorporation experiments were carried out to investigate whether Ang II induces proliferation of SK-UT-1 cells in the presence of growth factors. Cells, 5×10^4 /well, were cultured in 24 well-plates in normal medium containing 10% iFCS for 24 hrs and then 48 hrs in starved medium. The cells were then incubated with Ang II (10^{-7} M) in the presence or absence of the AT1 and AT2 receptor antagonists, losartan (10^{-5} M) and PD123177 (10^{-6} M) respectively for 16 to 20 hrs (overnight) in normal medium containing 10% iFCS or 1% insulin transferrin selenium (ITS). Cell counting and ^3H -thymidine incorporation (Fig 1-3 and Fig. 1-4) show that stimulation of AT1 or AT2 receptors did not significantly influence the growth factor-induced cell proliferation.

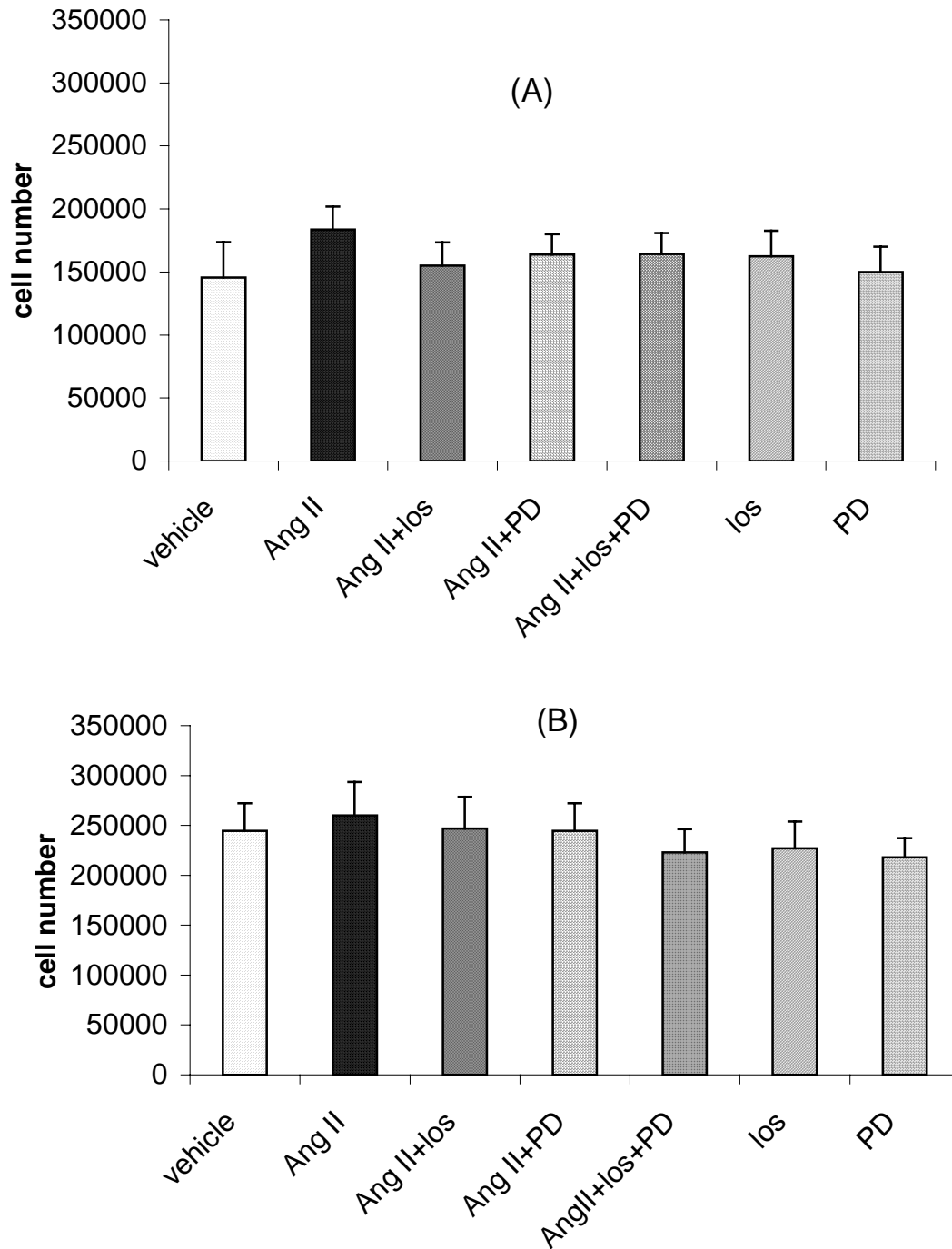


Fig 1-3. Effects of Ang II on the proliferation of SK-UT-1 cells cultured in the presence of growth factors. Quiescent SK-UT-1 cells cultured in medium containing (A) 10% iFCS or (B) 1% ITS were treated with Ang II (10^{-7} M); Ang II (10^{-7} M) + losartan (10^{-5} M); Ang II (10^{-7} M) + PD123177 (10^{-6} M) or Ang II (10^{-7} M) + losartan (10^{-5} M) + PD123177 (10^{-6} M) for 16-20 hrs. Cells were harvested by trypsin/EDTA solution and counted in a hemocytometer. The presented data are from a single experiment and representative of four independent experiments. Values represent means \pm SEM of n=12.

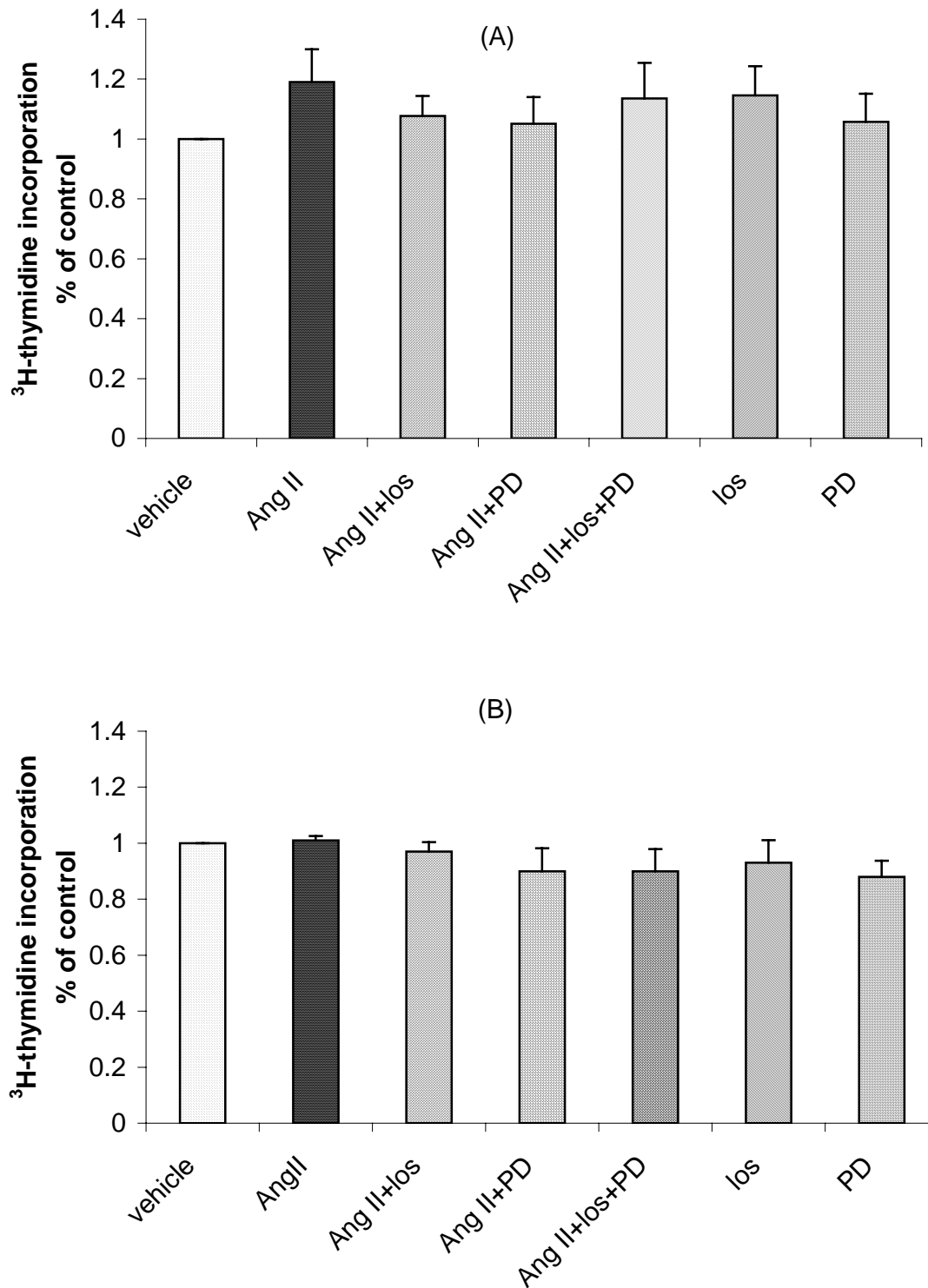


Fig 1-4. Effects of Ang II on [methyl,1,2-³H]-thymidine incorporation in SK-UT-1 cells cultured in the presence of growth factors. Quiescent SK-UT-1 cells cultured in medium containing (A). 10% iFCS or (B). 1% ITS were treated with Ang II (10^{-7} M); Ang II(10^{-7} M) + losartan (10^{-5} M); Ang II (10^{-7} M) + PD123177 (10^{-6} M) or Ang II (10^{-7} M) + losartan (10^{-5} M) + PD123177 (10^{-6} M) for 16-20 hrs. The presented data are from a single experiment and representative of four independent experiments. Values represent means \pm SEM of n=16.

The AT2 receptor has been demonstrated to be abundantly expressed upon high confluence in primary mesangial cells derived from normotensive Wistar-Kyoto rats (Goto et al., 1997). To study the effect of Ang II on SK-UT-1 cells cultured upon high confluence conditions, the same experimental protocol was used as in previous experiments. Cells were cultured in 24 well plates until they reached high confluence. Quiescent cells were then incubated with Ang II (10^{-7} M) in the presence or absence of the AT1 and AT2 receptor antagonists, losartan (10^{-5} M) and PD123177 (10^{-6} M), respectively for 16 to 20 hrs (overnight) in normal medium containing 10% iFCS. Fig 1-5 shows that Ang II had not significantly effect on proliferation of these cells.

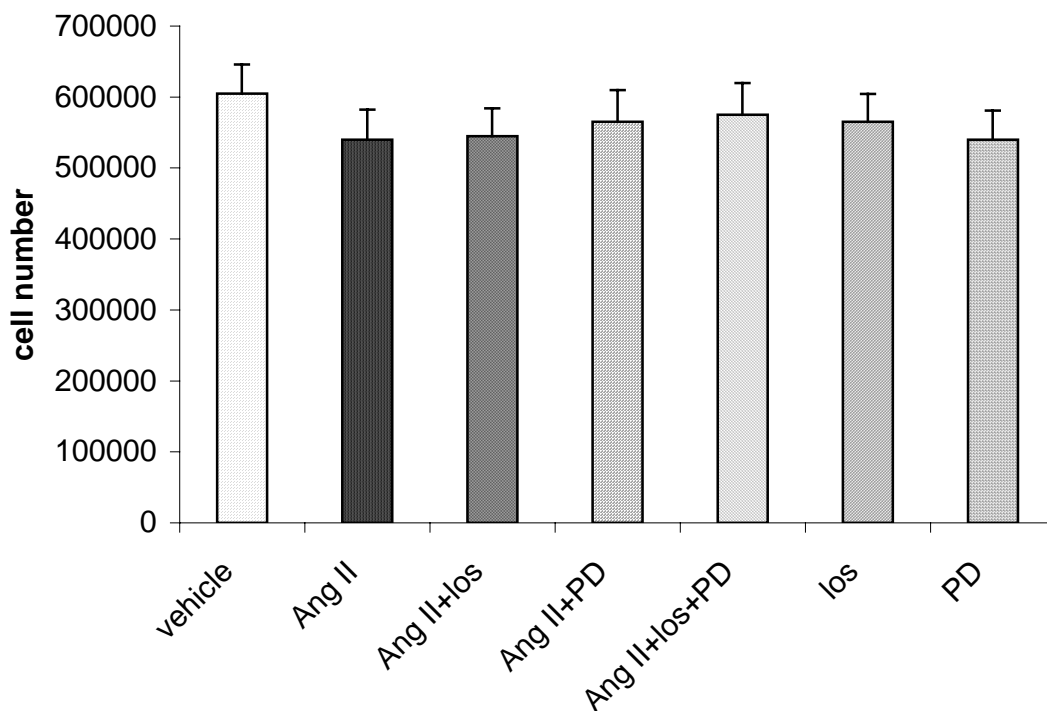


Fig 1-5. Effects of Ang II on the proliferation of SK-UT-1 cells cultured upon high confluence. SK-UT-1 cells were cultured until they reached high confluence. Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M); Ang II(10^{-7} M) + losartan (10^{-5} M); Ang II (10^{-7} M) + PD123177 (10^{-6} M) or Ang II (10^{-7} M) + losartan (10^{-5} M) + PD123177 (10^{-6} M) in culture medium containing 10% iFCS for 16-20 hrs. Cells were harvested by trypsin/EDTA solution and counted in a hemocytometer. The presented data are from a single experiment and representative of four independent experiments. Values represent means \pm SEM of n=12.

Taken together, these experiments clearly demonstrate that Ang II has no effect on the cell numbers or on the cell DNA synthesis in both, quiescent and proliferating SK-UT-1 cells.

Ang II and the expression of SM22, a differentiation marker, in SK-UT-1 cells

The following experiments were aimed to study whether Ang II can induce differentiation in leiomyosarcomas cells. The cells were cultured in normal medium until 70% confluence. Quiescent cells, obtained by serum deprivation for 48 hrs, were treated with Ang II (10^{-7} M) in the presence or absence of the AT1 and AT2 receptor antagonist, losartan (10^{-5} M) and PD123317 (10^{-6} M), respectively, for 1, 3 and 6 hrs in culture medium containing 1% ITS. Total RNA was extracted using GTC buffer according to the protocol by Chomczynski and Sacchi. RT-PCR was carried out using 5 μ g of total RNA. Fig 1-6 (A) shows that Ang II up-regulated the mRNA level of SM22 in SK-UT-1 cells only when the AT1 receptor was blocked by losartan. The effect was completely reversed by the addition of the AT2 receptor antagonist, PD1231777. One hour post-treatment of Ang II alone did not up-regulate SM22 mRNA when both receptor subtypes were stimulated by the peptide. The stimulatory effect of Ang II on the SM22 mRNA expression in the presence of the AT1 receptor antagonist could be detected only 1 hr after the peptide treatment. Three and 6 hrs post-stimulation with Ang II SM22 mRNA levels were equally induced in all experimental groups.

To test whether the activation of AT2 receptors exerts stimulatory effects on the SM22 mRNA expression at earlier time points, cells were stimulated with Ang II \pm losartan for 15, 30, 60 and 90 min. Fig 1-6 (B) shows that AT2 receptor activation already increased SM22 mRNA level after 15 min and the effect lasted up to 60 min. These results show that Ang II acting on its AT2 receptor up-regulates one of the VSMC-differentiation marker. Stimulation of AT1 receptors has no effect on the expression of this differentiation marker but reversed the AT2 receptor-mediated effect of the peptide.

(A)

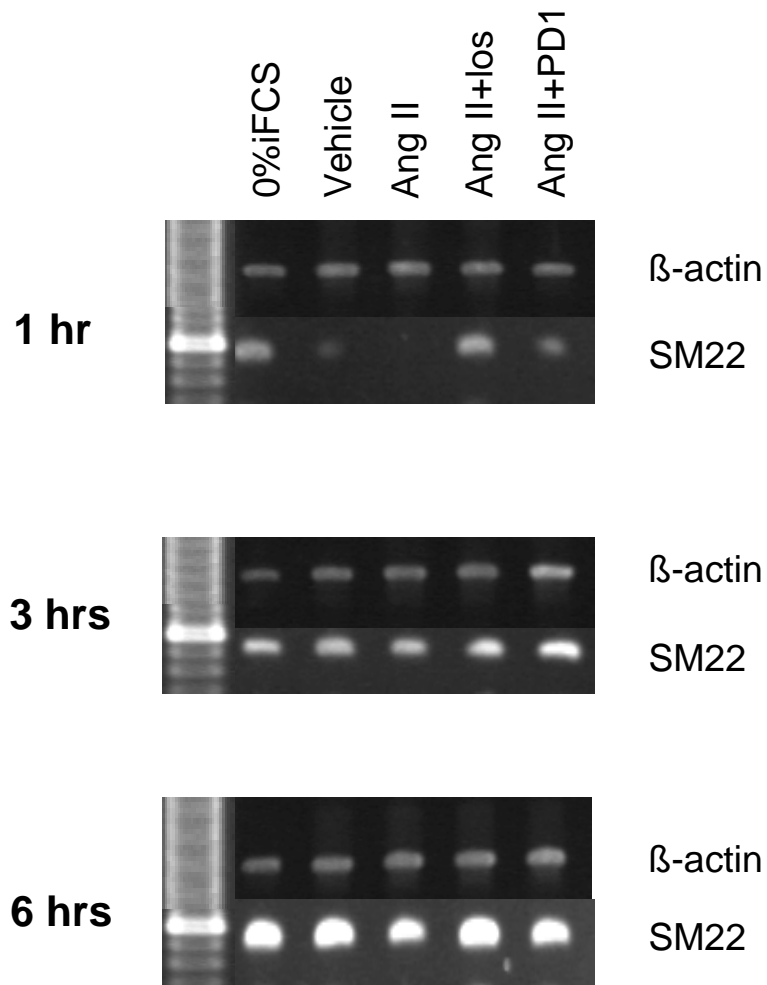


Fig 1-6. (A) and (B). See following page for the legend.

(B)

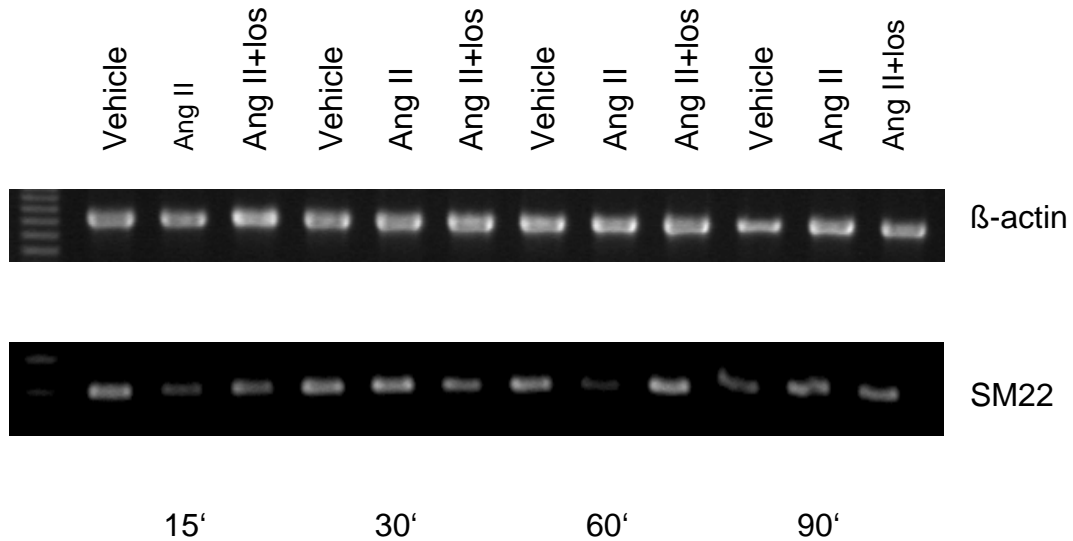


Fig 1-6. Differential expression of SM22 in SK-UT-1 cells after treatment with Ang II. Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M) \pm losartan (10^{-5} M) or PD123177 (10^{-6} M) for indicated time intervals in the presence of 1% ITS. Vehicle-treated cells served as a control. Total RNA was isolated from the cells and 5 μ g of total RNA were used for RT-PCR. Fifteen μ l of PCR products were loaded on 1.1% agarose gel and run at 90 V for 90 min. (A) SK-UT-1 cells were treated with Ang II in the presence or absence of losartan or PD123177 for 1, 3 and 6 hrs. (B) SK-UT-1 cell were treated with Ang II in the presence or absence of the AT1 receptor antagonist, losartan, for 15, 30 60 and 90 min.

Ang II and the expression of calponin in SK-UT-1 cells

Calponin is another smooth muscle differentiation marker which is additionally involved in the SMC contraction. To further investigate the role of Ang II in the differentiation in SMC, the effect of the peptide on the expression of calponin mRNA was studied. The protocol was identical to the protocol applied when the effects of Ang II on the SM22 mRNA expression were investigated. Fig1-7 shows that incubation of cells with Ang II for 1 hr considerably increased mRNA levels of calponin only when the AT1 receptor was inhibited, no effect was detectable following incubation with the peptide alone for 3 or 6 hrs. Again, the effect of Ang II was AT2 receptor-mediated since PD123177 completely abolished the effect of Ang II on the mRNA expression of calponin. These results indicate that activation of the AT2 receptor up-regulates the transcription of calponin. Fig 1-8 summarizes the data from two independent experiments demonstrate that calponin was up-regulated by Ang II only when the AT1 receptor was blocked by losartan. However, the effect can be detected only after peptide stimulation for 1 hr.

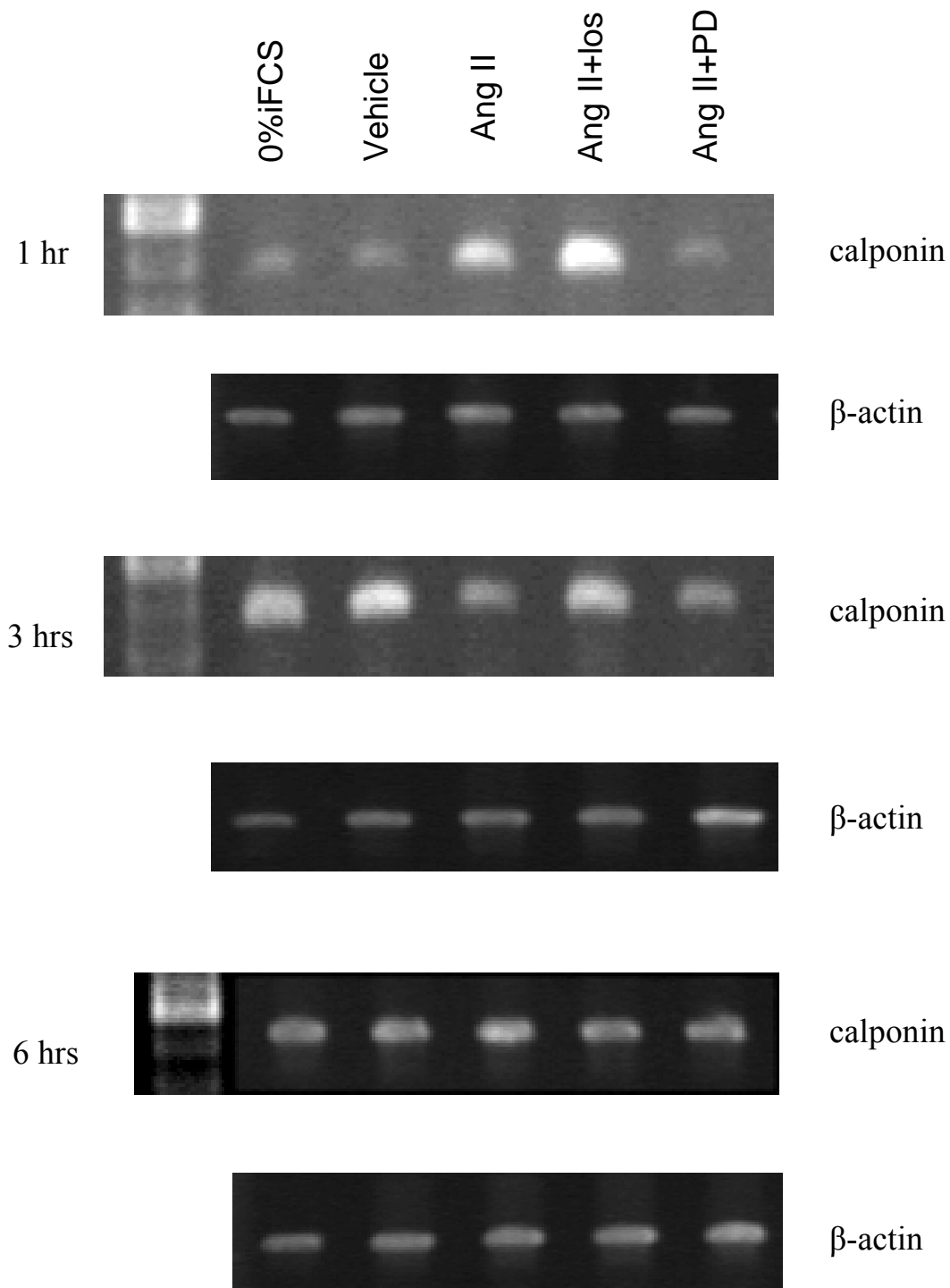


Fig 1-7. Differential expression of calponin in SK-UT-1 cells after treatment with Ang II. Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M) \pm losartan (10^{-5} M) or PD123177 (10^{-6} M) for indicated time intervals in the presence of 1% ITS. Vehicle-treated cells served as a control. Total RNA was isolated from the cells and 5 μ g of total RNA were used for RT-PCR. Fifteen μ l of PCR products were loaded on 1.1% agarose gel and run at 90 V for 90 min. SK-UT-1 cells were treated with Ang II in the presence or absence of its receptor antagonists for 1, 3 and 6 hrs.

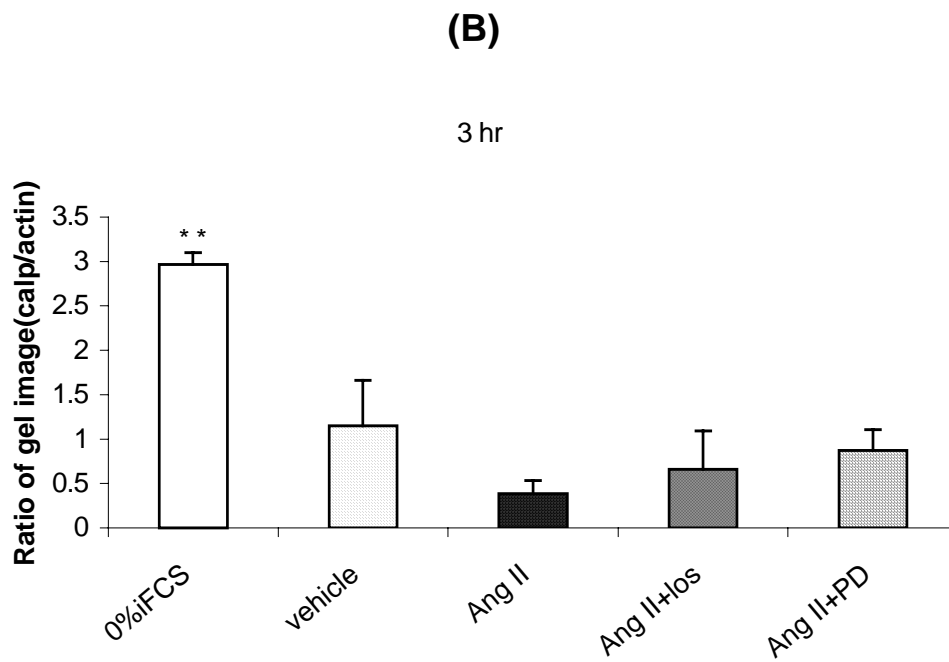
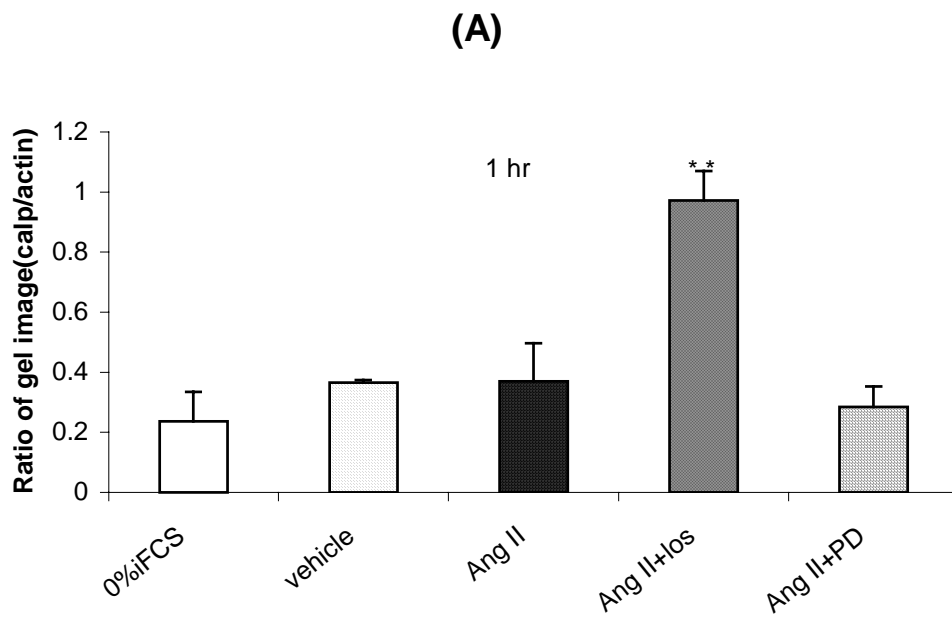


Fig 1-8. (A) and (B). See following page for the legend

(C)

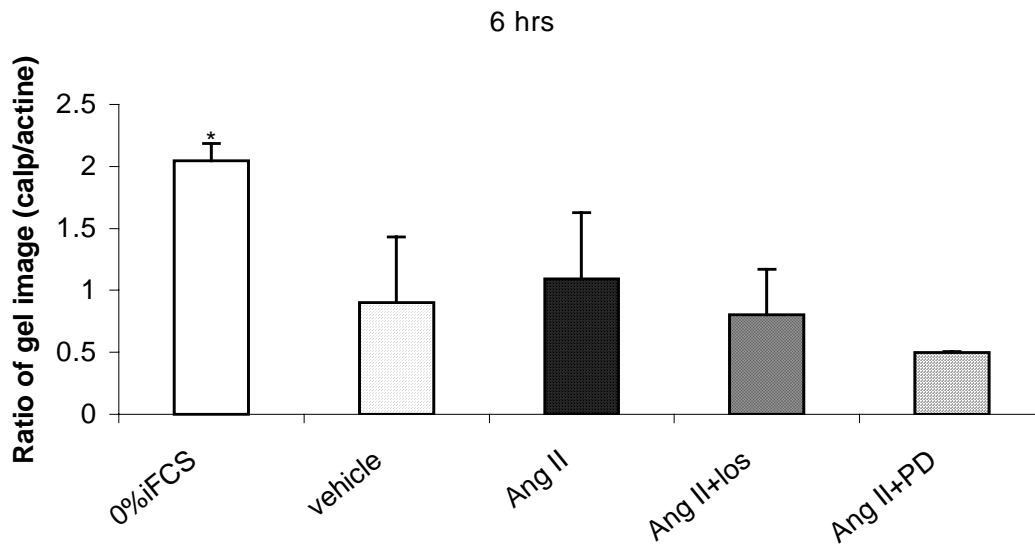


Fig 1-8. The densitometric quantification of agarose gel image for PCR products (calponin/beta-actin). Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M), Ang II (10^{-7} M) + losartan (10^{-5} M) and Ang II (10^{-7} M) + PD123177 (10^{-6} M), in the presence of 1% ITS. Total RNA was isolated at 1, 3 and 6 hrs post-treatment. Vehicle- treated cells were used as control. Results obtained in two independent experiments were pooled. Values represent means of n=2.

Effect of Ang II on calponin protein expression

In the previous studies, it was demonstrated that Ang II, upon activation of AT2 receptors, stimulates the expression of calponin mRNA. In this part, we investigated the effects Ang II on the calponin expression at the translation level, i.e. we quantified protein levels of calponin after stimulation with Ang II. The cells were cultured in the normal medium until 70% confluence and then the cells were serum deprived for 48 hrs to obtain quiescence. The cells were then treated with Ang II (10^{-7} M) in the presence or absence of angiotensin receptor antagonists, losartan (10^{-5} M) and PD123177 (10^{-6} M), respectively, for 3, 6, 9, 12, 24 hrs in 1% ITS culture medium. The crude proteins were harvested with cell lysis buffer and 30 μ g of total proteins was loaded on 10% polyacrylamide gel. Western blotting was carried out with human antibody against calponin (dilution 1:2500) followed by mouse peroxidase-linked anti-human antibody (dilution 1:2000). Fig 1-9 shows that Ang II increased the calponin protein level at only one time point (9 hrs) and only when the AT1 receptor was inhibited by losartan. The effect was not detectable at earlier or later time points.

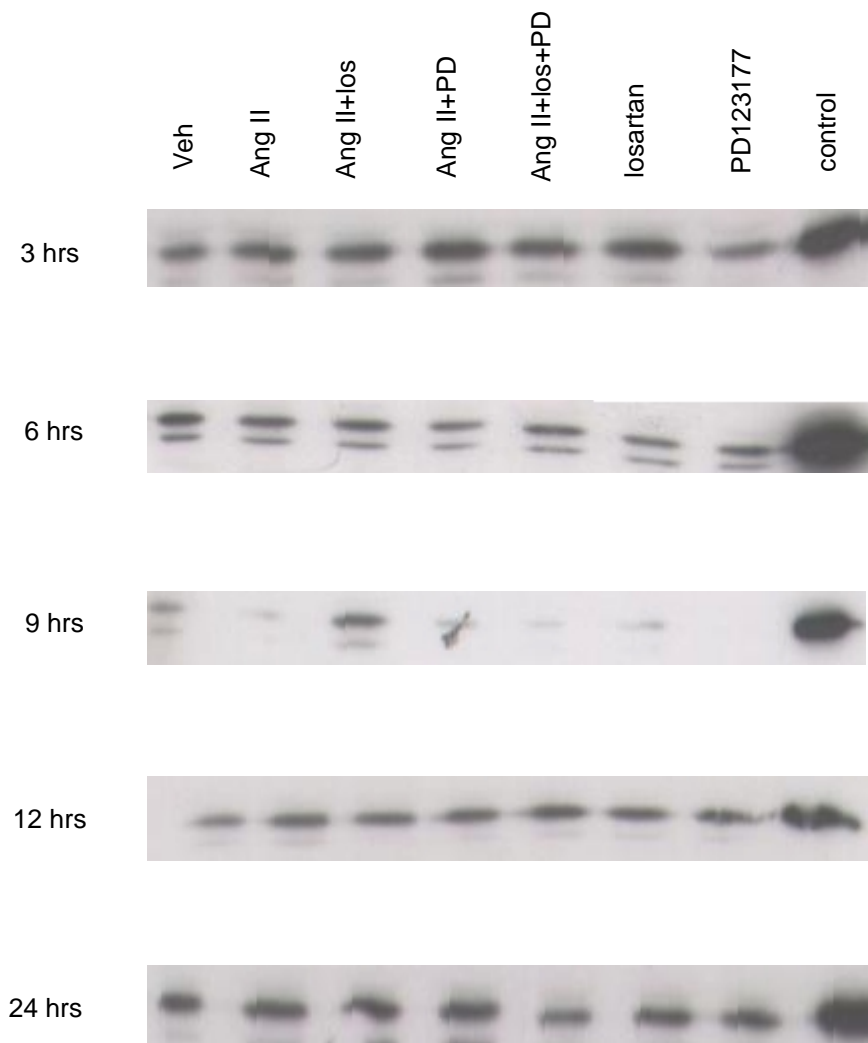


Fig 1-9. Effect of Ang II on calponin protein expression. Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M) \pm losartan (10^{-5} M) and/or PD123177 (10^{-6} M) in the presence of 1% ITS for 3, 6, 9, 12 and 24 hrs. Vehicle-treated cells were negative controls and human umbilical smooth muscle cells served as positive controls. Crude proteins were harvested in cell lysis buffer containing 50 mM Tris-HCl (pH 8.0), 0.25 M NaCl; 0.5% NP-40; 1 mM PMSF; 1 μ g/ml aprotinin; 1 μ g/ml leupeptin; 20 μ g/ml TPCK. 30 μ g of total proteins were loaded on 10% SDS polyacrylamide gel and run at 150 V for 65 min. Proteins were then transferred on PVDF membrane and incubated with the first antibody followed by incubation with horseradish peroxidase-labelled second antibody. Three to 5 independent experiments were carried out.

Ang II and apoptosis-induction in SK-UT-1 cells evaluated by TUNEL method

Recent findings indicate that Ang II acting on the AT2 receptor not only promotes cell differentiation, but can also induce apoptosis. In the following part, it was studied whether SK-UT-1 cells increasingly undergo apoptosis when they are exposed to Ang II. TUNEL and DNA-ladder methods were used.

In the TUNEL experiment, SK-UT-1 cells (5000/well) grew in 24 well-plates in normal medium for 72 hrs and then they were serum deprived for 48 hrs to obtain quiescence. These cells were then treated with Ang II (10^{-7} M) in the presence or absence of losartan (10^{-5} M) in 1% ITS medium for 24, 48, and 72 hrs to obtain the optimal time point for the experiment. Every 12 hrs, fresh Ang II and losartan were added to the culture medium. The rate of apoptotic events in the cells was investigated by the TUNEL method 24, 48 and 72 hrs after the onset of treatment of cells with Ang II or Ang II + losartan. In the second experiment, cells were treated with Ang II alone, or with Ang II combined with losartan or PD123177 (10^{-6} M) for 48 hrs. Cells incubated with DNase served as a positive control whereas cells untreated with labeling enzyme for the first antibody served as a negative control (see Materials and Methods). Another control group represented cells treated with vehicle. Fig 1-10 shows that apoptotic cells could be detected after treatment with Ang II plus losartan. Apoptosis was most pronounced after 48 hrs of stimulation with Ang II + losartan and diminished after 72 hrs of treatment. The results presented in Fig 1-10 and Fig 1-11 show that Ang II induced apoptosis in SK-UT-1 cells when AT1 receptors were blocked, while in the presence of the AT2 receptor antagonist, no apoptosis was detectable. These data clearly indicate that the Ang II effect on apoptosis is AT2 receptor-mediated.



Fig 1-10 . See page 70 for legend.

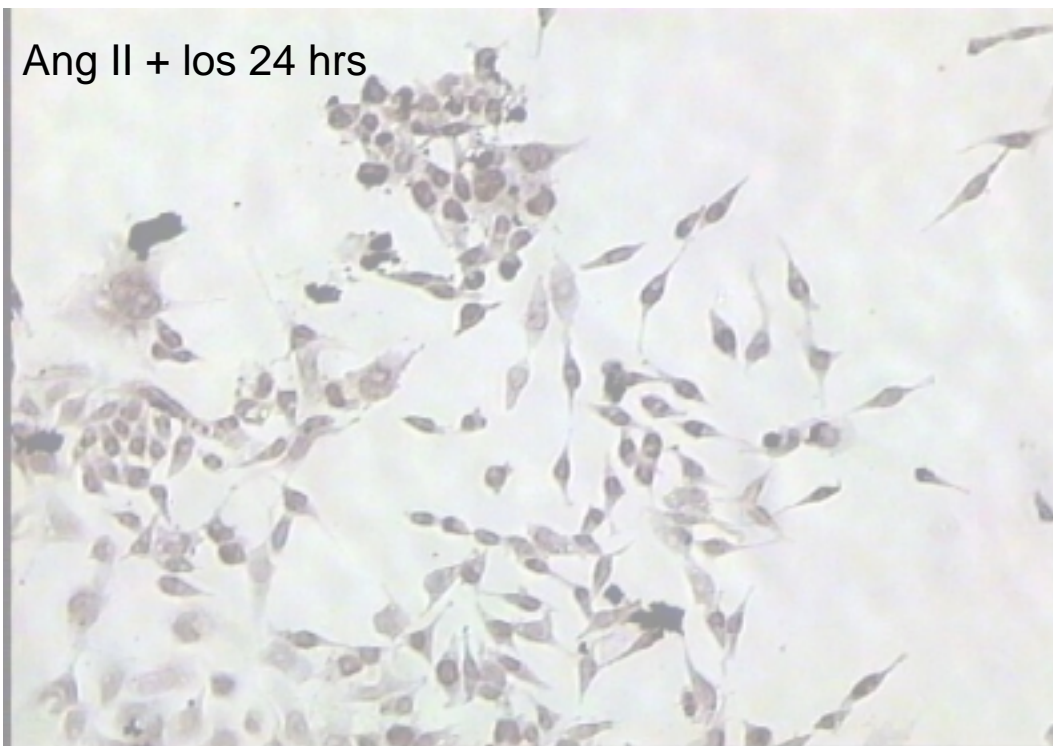
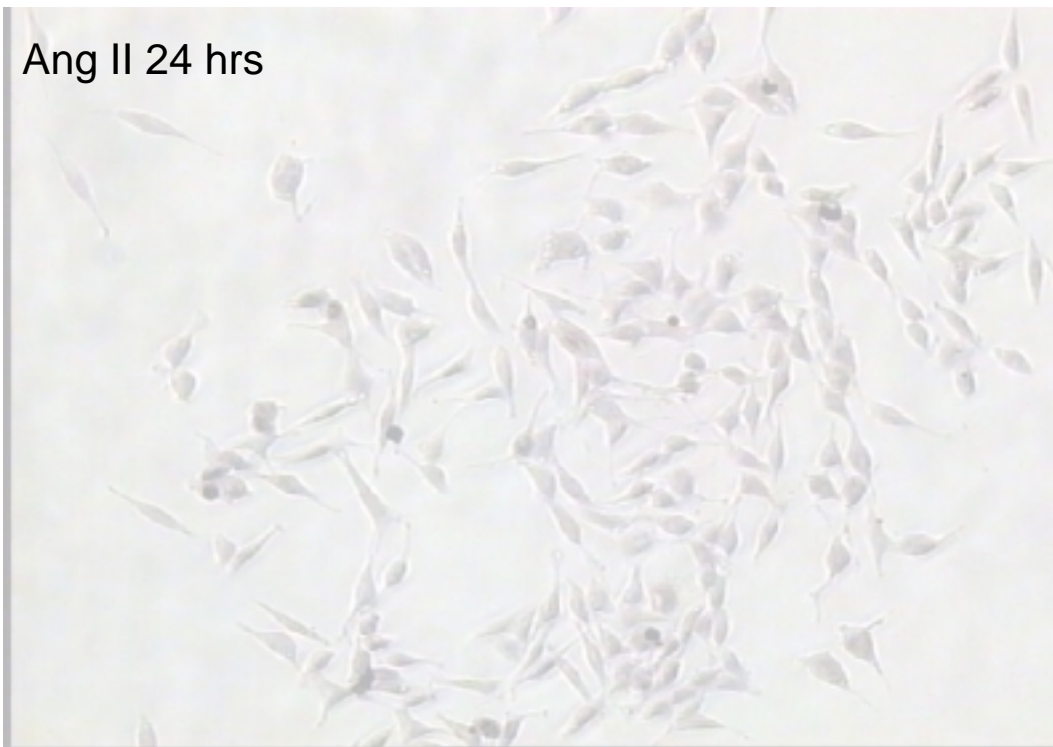


Fig 1-10. See page 70 for legend.



Fig 1-10. See page 70 for legend

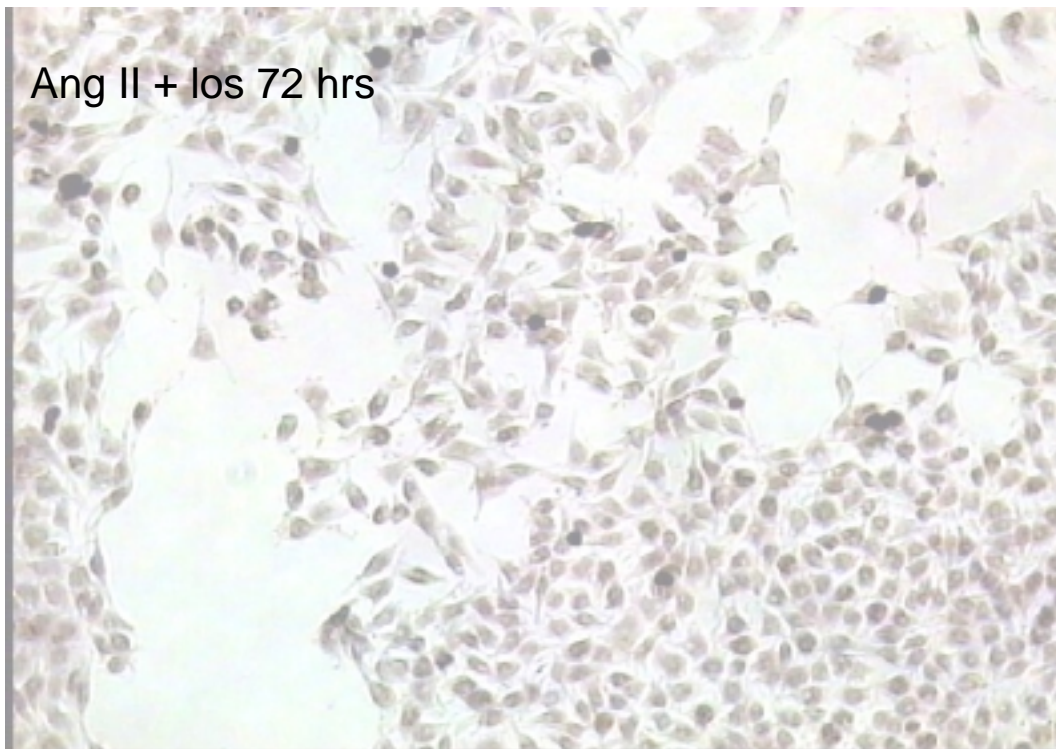
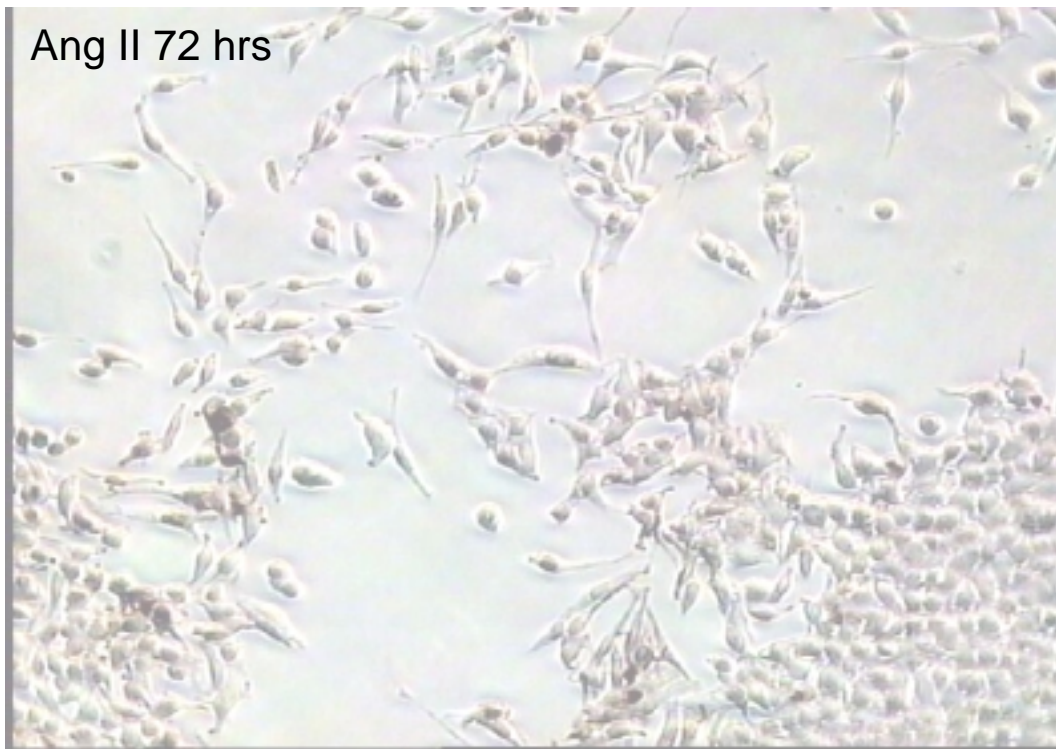


Fig 1-10. Time-dependent induction of apoptosis post treatment with Ang II in SK-UT-1 cells Quiescent SK-UT-1 were treated with Ang II (10^{-7} M) \pm losartan (10^{-5} M) for 24, 48 and 72 hrs. Treated cells were subjected to TUNEL staining. DNase treated cells serves as a positive control. Nucleotides of stained with DAB-substrate were identified as apoptotic cells.

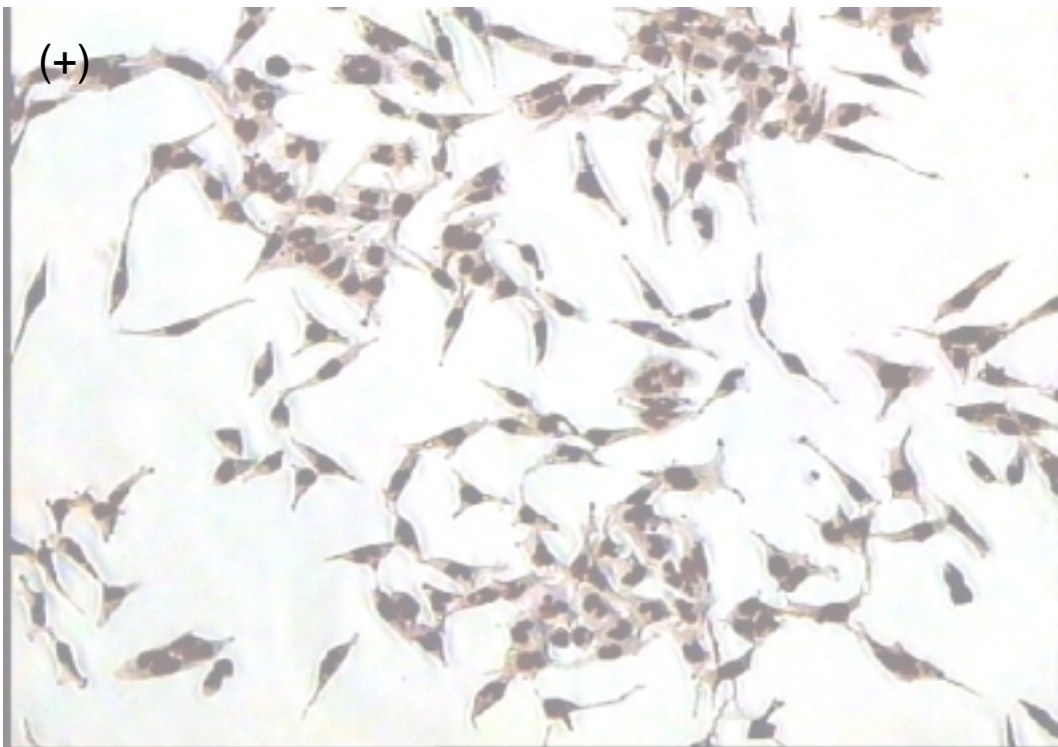


Fig 1-11. See following page for legend.

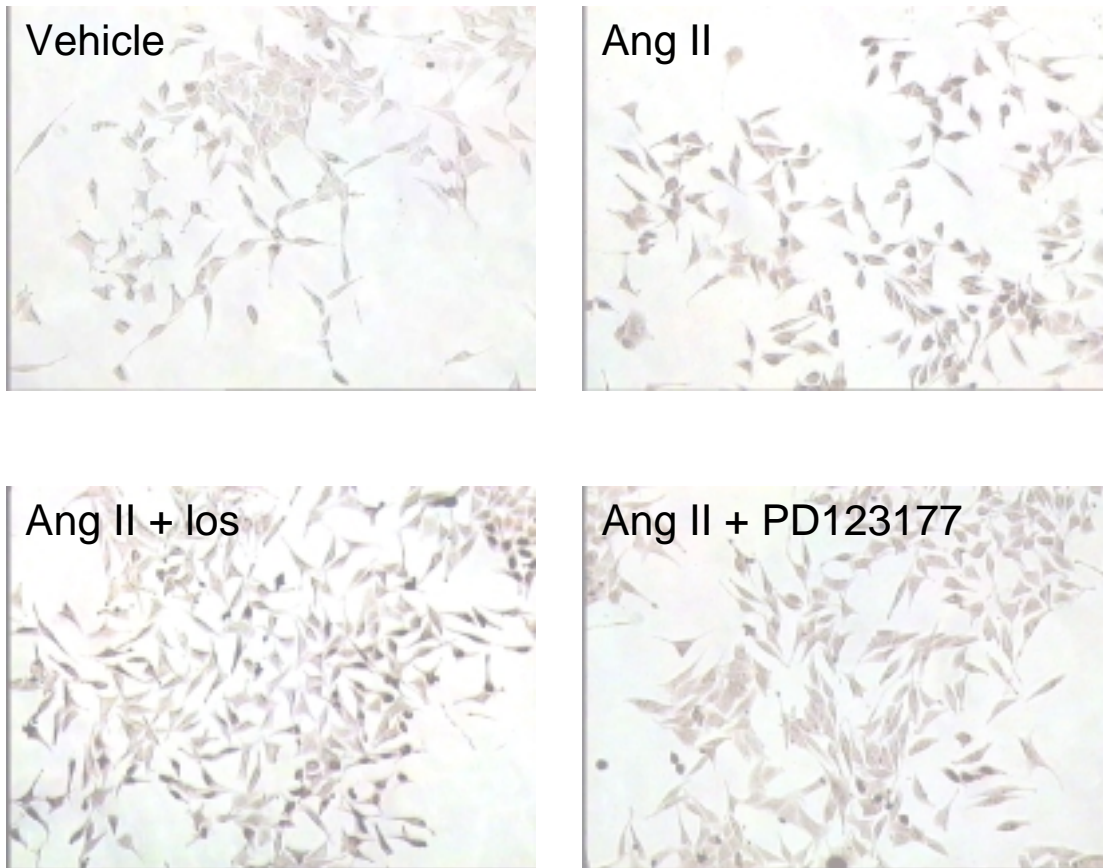


Fig 1-11. Ang II induced apoptosis in SK-UT-1 cells was AT2 receptor-mediated. Quiescent SK-UT-1 cells were treated with Ang II (10^{-7} M) \pm losartan (10^{-5} M) or PD123177 (10^{-6} M) per 12 hrs till 48 hrs. Cells were fixed with 4% paraformaldehyde (pH 7.4) and subjected to TUNEL staining. Cells treated with DNase were used as a positive control and untreated as a negative control. Three independent experiments were carried out.

Ang II and apoptosis-induction in SK-UT-1 cells evaluated by DNA-ladder method

The protocol was identical to the previous TUNEL protocol. To obtain the optimal time point for the experiment, quiescent cells were treated with Ang II (10^{-7} M) in the presence or absence of losartan (10^{-5} M) in 1% ITS medium every 12 hrs. The cells were collected for DNA isolation at 24, 36, and 48 hrs after the onset of stimulation with the ligands. Treatment of quiescent SK-UT-1 cells with Ang II + losartan for 24, 36 and 48 hrs induced fragmentation of DNA; the effect was most pronounced at 36 hrs (Fig 2-11 A). In the second experiment, cells were treated with Ang II alone, or with Ang II combined with losartan or PD1123177. Cells treated with vehicle or with angiotensin receptor antagonists alone served as negative controls. The Ang II induced apoptosis in SK-UT-1 cells was most pronounced after 36 hr of peptide stimulation together with losartan. This effect was mediated by the AT2 receptor, since the fragmentation of DNA could be detected only when the AT1 receptor was blocked whereas no fragmentation of DNA was observed in the presence of the AT2 receptor antagonist (Fig 1-12 B).

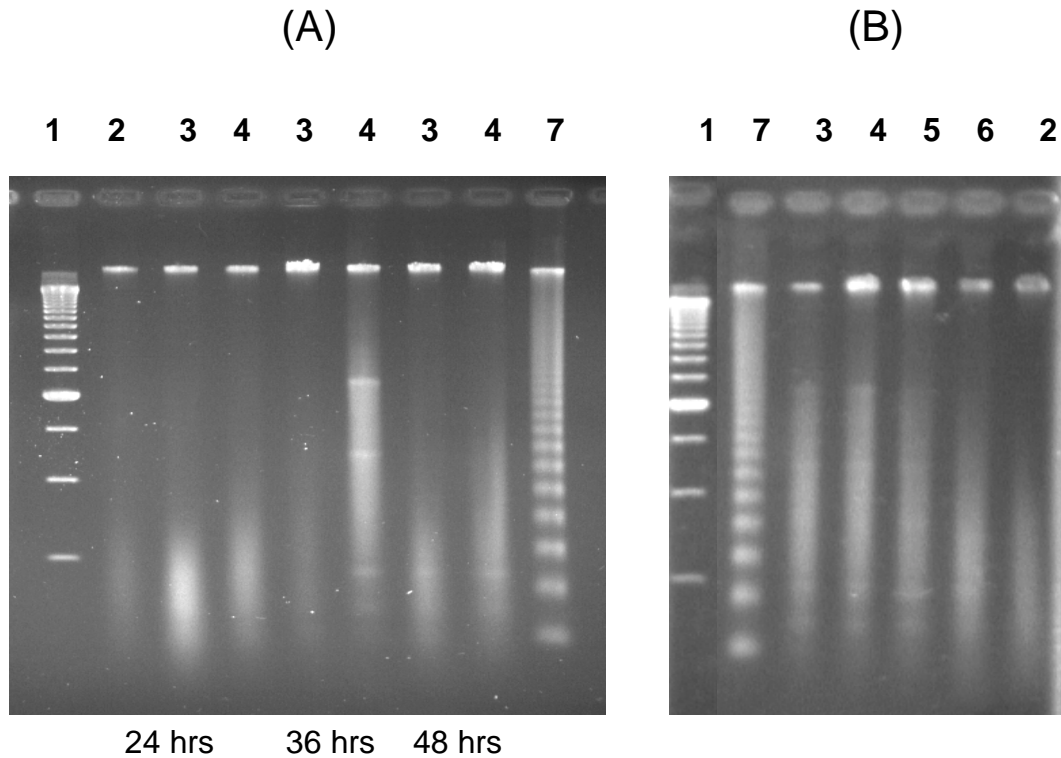


Fig 1-12 Ang II and apoptosis-induction in SK-UT-1 cells evaluated by the DNA-ladder method. Quiescent SK-UT-1 cells were treated every 12 hrs with Ang II (10^{-7} M) +/- losartan (10^{-5} M) and/or PD123177 (10^{-6} M) in the presence of 1% ITS. The cells were harvested after 24, 36 and 48 (A) or 36 (B) hrs of incubation with angiotensin receptor ligands. After DNA had been isolated (see Material and Method section), 3 μ g of total DNA from each sample was loaded on 1% of agarose gel and run at 60 V for 3 hrs. Apoptotic cells provided in the "Apoptosis DNA-ladder Kit" served as positive control for the DNA isolation. Four independent experiments were carried out. Explanation of numbers in Fig.1-12: 1. DNA marker; Treatment with: 2. vehicle; 3. Ang II; 4. Ang II + losartan; 5. Ang II + PD123177; 6. Ang II + losartan + PD123177; 7. positive control.

Results (section II)

AT2 receptor induced Zfhyp expression in CEC

Initial experiments carried out in our laboratory have demonstrated that *Zfhyp* mRNA is increasingly expressed in PC12W cells when these cells are exposed to epidermal growth factor (EGF) (25ng/ml) ± Ang II (10^{-7} M). *Zfhyp* was induced after 1 hr but down-regulated after 6 hrs of Ang II stimulation. Subsequent experiments have shown Ang II causes a rapid induction of *Zfhyp* mRNA and this effect is abolished in the presence of the selective AT2 receptor antagonists, PD123177. These initial experiments indicated that Ang II may regulate the growth factor-induced *Zfhyp* expression in PC12W cells, which express only the AT2 receptor. However, they have not answered the question whether Ang II via the AT2 receptor could directly regulate *Zfhyp* in the absence of growth factor in cells of vascular origin.

In the present experiments, the gene expression of *Zfhyp* in rat CEC to study the AT2 receptor-related gene expression in cells of vascular origin in the presence or absence of growth factor basic fibroblast growth factor (bFGF) was investigated. In addition, these cells express both Ang II receptor subtypes which allowed to study the potential role of the AT1 and the AT2 receptors in *Zfhyp* expression. The expression of both receptor subtypes in CEC was verified prior to the experiments by RT-PCR (Stoll et al., 1995).

CEC were isolated from male Wistar rats. In the first set of experiments. The Ang II-induced effects on *Zfhyp* mRNA expression in the CEC over a time course of 1 to 6 hrs were investigated. In quiescent CEC, 25 ng/ml bFGF stimulated *Zfhyp* mRNA up to 10 fold (positive control) (Fig2-1). Ang II (10^{-7} M), (3 hrs exposure) did not induce any *Zfhyp* mRNA when both receptor subtype were accessible. However, after pre-treatment with the selective AT1 receptor antagonist, losartan (10^{-5} M), stimulation with Ang II resulted in a marked *Zfhyp* mRNA expression, an effect that even exceeded the effect induced by bFGF (27 fold). On the contrary, in the presence of the AT2 receptor antagonist, PD123177 (10^{-6} M), no *Zfhyp* mRNA expression was detectable (Fig 2-1).

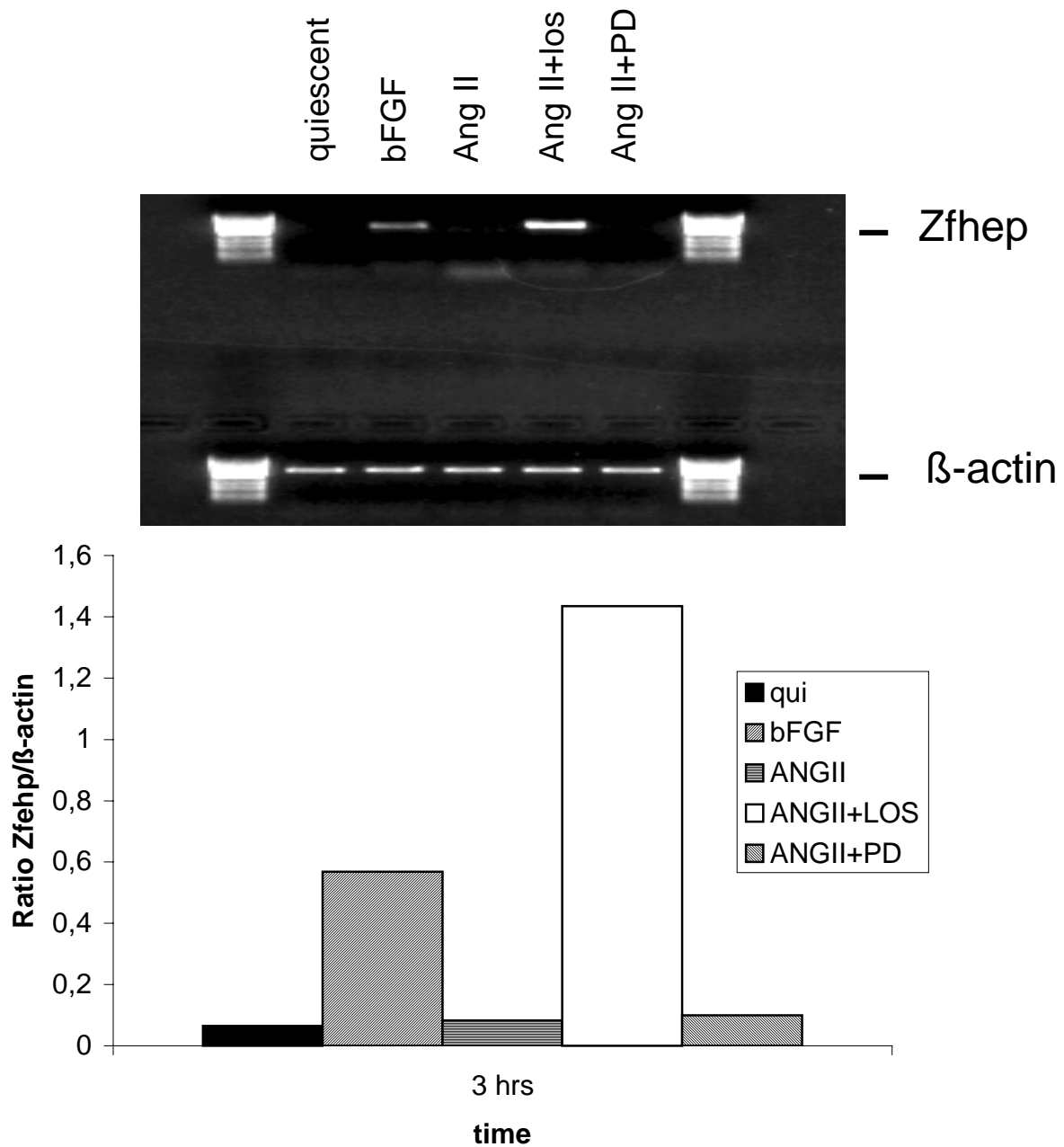


Fig 2-1. Differential expression of *Zfhep* mRNA in CEC exposed to Ang II +/- losartan or PD123177. Total RNA was isolated from quiescent CEC 3 hrs post stimulated with Ang II (10^{-7} M) +/- losartan (10^{-5} M) or PD123177 (10^{-6} M). An amount of 5 μ g of total of RNA was used for RT-PCR. 15 μ l of PCR product from each sample were loaded on 1.1% agarose gel and run at 90V for 90 min. CEC treated with 25 ng/ml bFGF used as positive control for *Zfhep* mRNA expression.

Effect of Ang II on Zfhhep mRNA expression in CEC pre-stimulated with bFGF.

Previous findings in our laboratory have demonstrated that Ang II significantly inhibits the proliferation of bFGF-stimulated CEC in a dose-dependent manner, and this effect can be prevented by PD 123177, whereas losartan was ineffective (Stoll et al., 1995). In the present study it was attempted to investigate whether the previously described antiproliferative effect of the AT₂ receptor in these cells was accompanied by changes in the expression of *Zfhhep* mRNA under identical experimental conditions. The experimental protocols used in the previous and the present study were identical. Shortly after pretreatment CEC with bFGF for 16 hrs, the cells were exposed to Ang II (10^{-7} M) +/- losartan (10^{-5} M) or PD 123177 (10^{-6} M) for 1, 3 and 6 hrs. Fig 2-2 (A) shows representative data from RT-PCR in CEC. Fig 2-2(B) summarizes the data from 5-7 independent experiments. Compared to quiescent controls, *Zfhhep* mRNA levels were no longer induced in these cells 16 hrs post-stimulation with bFGF (Fig 2-2). Furthermore, Ang II alone did not markedly alter *Zfhhep* mRNA expression in bFGF-treated cells, 1 and 3 hrs post-stimulation. However, in the presence of the AT₁ receptor antagonist, losartan, a significant increase in *Zfhhep* mRNA was observed up to 3 hrs post-stimulation with the peptide ($p < 0.01$ vs bFGF + Ang II), an effect that was reversed by the addition of the AT₂ receptor antagonist PD123177 ($p < 0.01$ vs bFGF + Ang II + PD123177 and $p < 0.05$ vs bFGF + Ang II + losartan + PD123177). Stimulation of the AT₁ receptor, did not affect *Zfhhep* mRNA expression in the presence of bFGF (Fig 2-2). In addition, Northern Blot analysis was used to verify the AT₂ receptor-mediated induction of *Zfhhep* mRNA in CEC. Although the expression level of *Zfhhep* was very low and the bands after Northern hybridization were weak, an induction of *Zfhhep* was observed after AT₂ stimulation (bFGF + Ang II + losartan) supporting the RT-PCR data. The AT₂ receptor-mediated induction of *Zfhhep* was again an early (1 hr) and transient phenomenon. It is noticeable that the degrees of *Zfhhep* expression in CEC treated only with bFGF and in non-treated quiescent cells were identical (Fig 2-3).

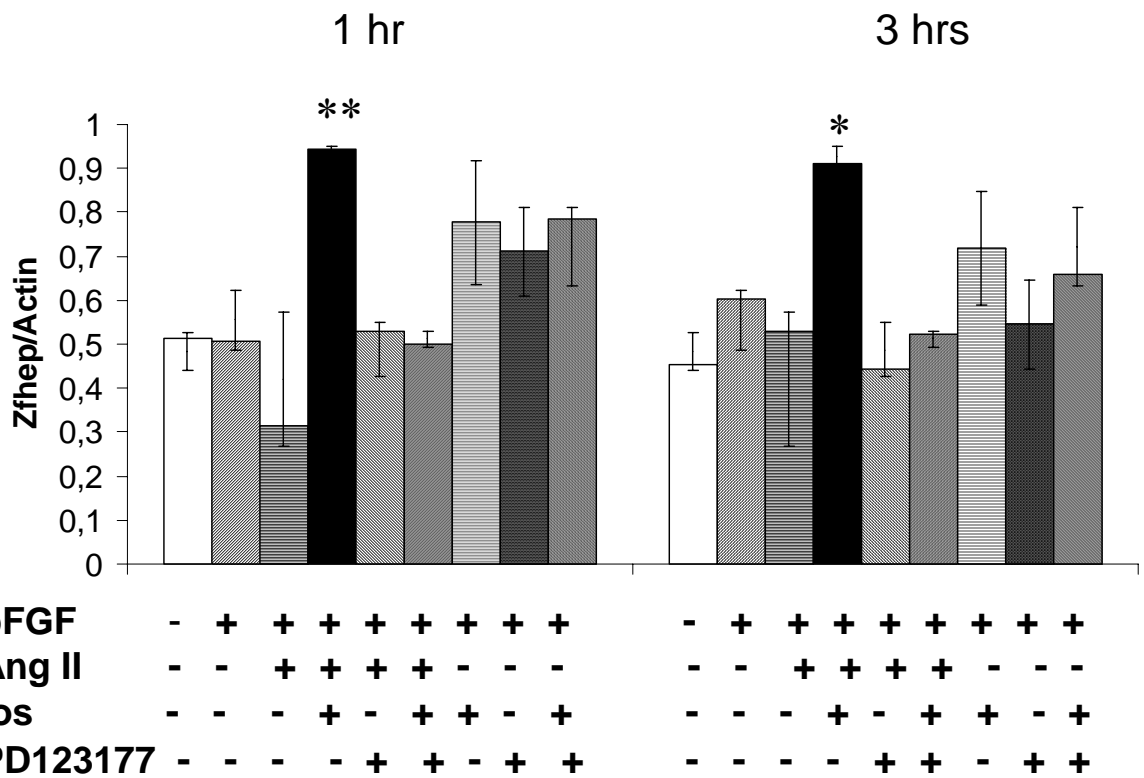
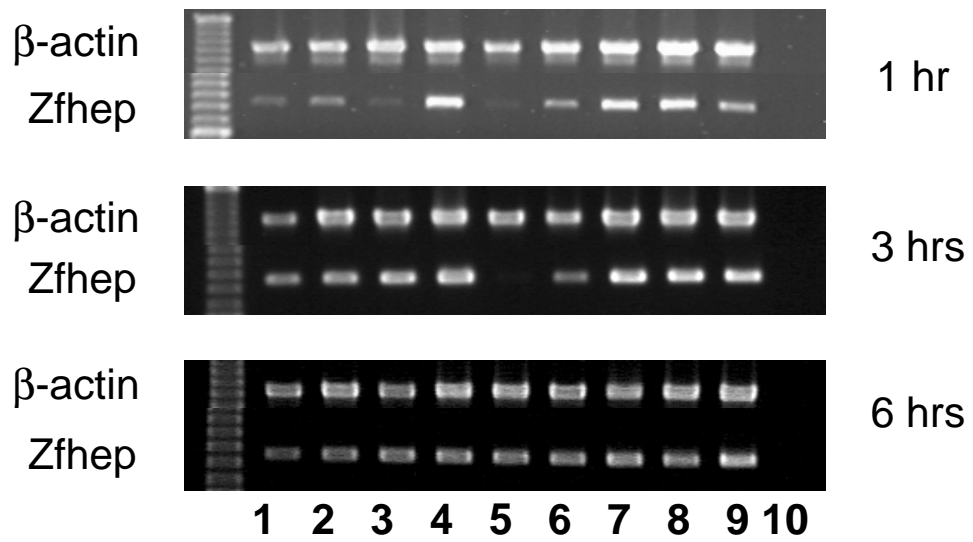


Fig 2.2 See the following page for the legend.

Fig 2-2. Effect of Ang II on the expression of *Zfh*ep in CEC. Cells were pre-stimulated (16 hrs) with bFGF (25 ng/ml). RNA was isolated 1, 3 and 6 hrs post stimulation with Ang II (10^{-7} M) +/- losartan (10^{-5} M) and/or PD123177 (10^{-6} M). A). representative gel images for *Zfh*ep mRNA expression for 1, 3 and 6 hrs post-stimulation. 1: quiescent cells, 2: vehicle, 3: Ang II, 4: losartan + Ang II, 5: PD123177 + Ang II, 6: losartan + PD123177 + Ang II, 7: losartan, 8: PD123177, 9: losartan + PD123177, 10: H₂O control. B). Graph showing the changes in expression based on densitometric analysis. 2-3 fold induction of *Zfh*ep mRNA in the presence of Ang II + losartan at 1 and 3 hrs post-stimulation was detected (n=7 for 1 hr, n=5 for 3 hrs). ** time point 1 hr: p<0.01 vs Ang II, p<0,01 vs Ang II + PD123177 and p< 0.05 vs Ang II + losartan + PD123177. * time point 3 hrs: p<0.05 vs Ang II, p<0.05 vs Ang II + PD123177, p<0.05 vs Ang II + losartan + PD123177.

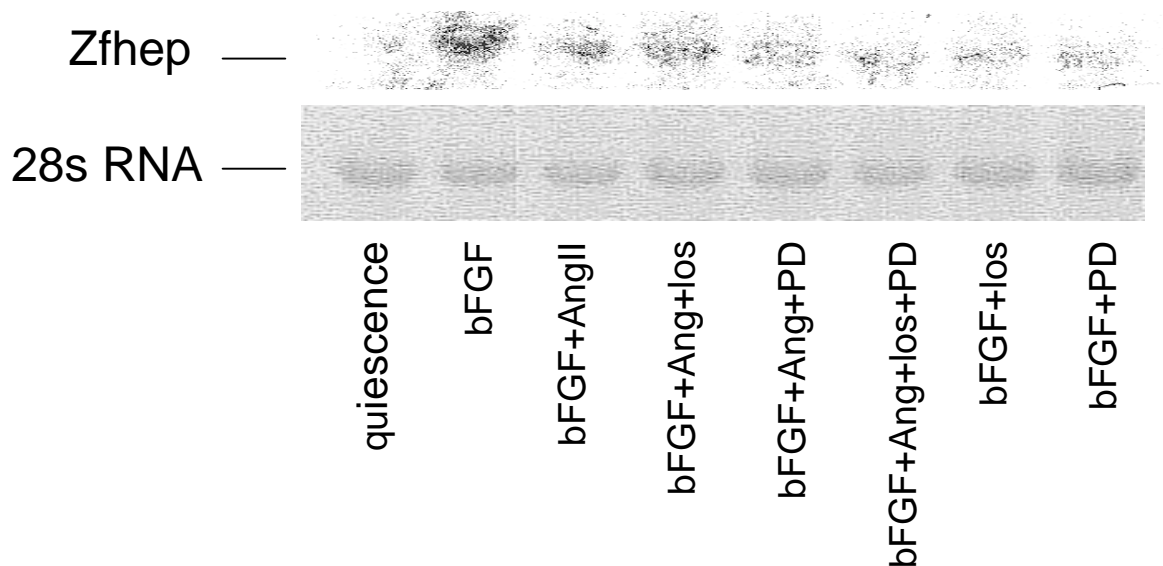


Fig 2-3 *Zfh*ep mRNA expression in CEC, Northern Blot analysis. The expression of *Zfh*ep mRNA in CEC pre-stimulated with bFGF in response to Ang II (10^{-7} M) +/- losartan (10^{-5} M) or PD 1231777 (10^{-6} M) or losartan and PD 123177 alone, was investigated 1 hr after the stimulation with the angiotensin receptor ligands. Total RNA was isolated from the same samples as described above. 10 μ g of total RNA from each sample were loaded on 1% of agarose gel for RNA isolation and run at 18 mA in 1 X MOPS buffer for 17.5 hrs (see Material and Methods section). 28s RNA served as controls for equal loading of lanes.

Results (section III)

Ang II-induced differentiation in PC12W cells: Involvement of NO

In homogenates from the aorta of SHR, Ang II increased cGMP levels after pre-treatment with losartan to block the AT1 receptor, indicating that this effect is AT2 receptor-mediated (Gohlke et al., 1998). Correspondingly, inhibition of the AT2 receptor completely abolished the Ang II-induced cGMP production. However, blockade of the bradykinin B2 receptor by icatibant also inhibited this effect. These findings point to a link between AT2 receptors and NO production through B2 receptor activation, since activation of B2 receptors increases NO production which, in turn, enhances the activity of soluble guanylate cyclase to produce cGMP (Gohlke et al., 1998). Ang II induces differentiation in PC12W cells, which exclusively express the AT2 receptor (Meffert et al., 1996). In the last part of the study, the possible contribution of the BK/NO cascade to the differentiation in PC12W cells induced by activation of AT2 receptors was investigated.

Quiescent PC12W cells, cultured in collagen A pre-treated dishes, were stimulated daily with NGF (positive control) or with Ang II (10^{-7} M) in the presence or absence of the NO synthase (NOS) inhibitor, L-NAME (10^{-5} M), for 6 days. The degree of cell differentiation was evaluated by counting of neurite outgrowths per 100 cells (see Materials and Methods). Only the numbers of medium length neurites are presented in this section.

As expected, both, NGF and Ang II stimulated neurite outgrowth in quiescent PC12W cells (Fig. 3-1). The Ang II-induced differentiation in PC12W cells was quantitatively similar to that induced by NGF. In contrast to the previous report (Meffert et al., 1996), NGF (0.5 ng/ml) did not potentiate the Ang II-induced cell differentiation. NOS synthase inhibitor, L-NAME, completely blocked the effect on differentiation of PC12W cells induced by Ang II in the presence or absence of NGF (0.5 ng/ml) (Fig 3-2).

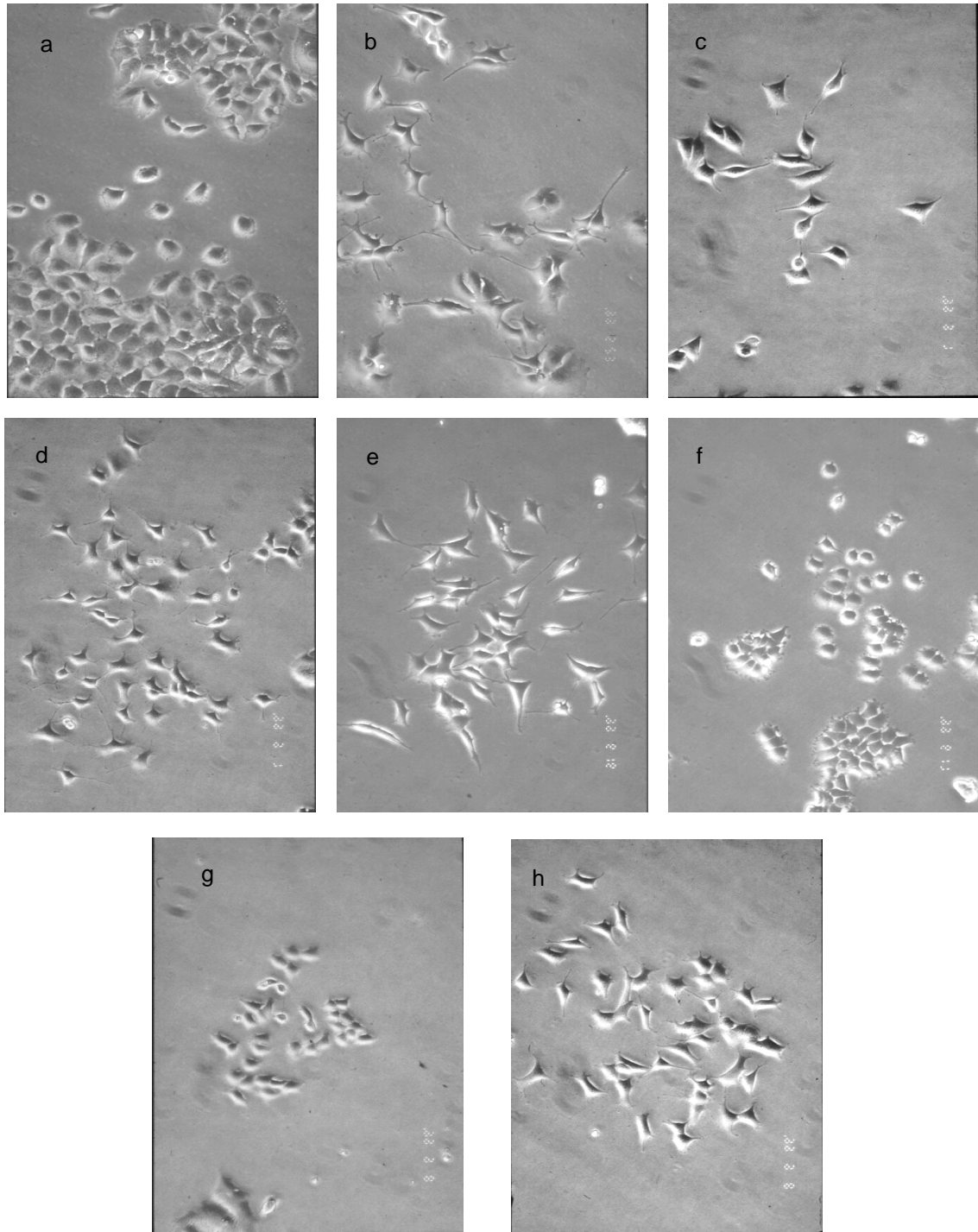


Fig. 3-1. Morphological change in PC12W cells treated with various differentiation inducers. (a). vehicle treatment, (b). NGF (50ng/ml), (c). NGF (0.5 ng/ml), (d). Ang II (10^{-7} M), (e). NGF (0.5 ng/ml) + Ang II (10^{-7} M), (f). NGF (0.5 ng/ml) + Ang II (10^{-7} M) + L-NAME (10^{-5} M), (g). Ang II (10^{-7} M) + L-NAME (10^{-5} M), (h). sodium nitroprusside (SNP, 10^{-7} M).

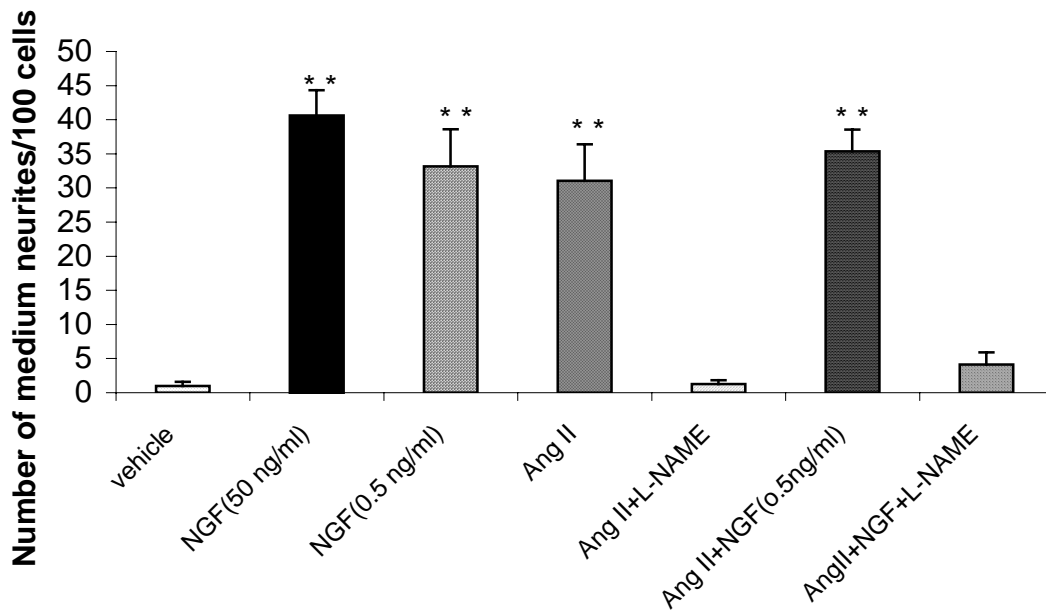


Fig 3-2 Effect of NO synthase inhibition on the Ang II-induced differentiation in PC12W cells. PC12W cells were planted on collagen A pre-treated dishes and serum deprived for 48 hrs to obtain quiescence. Ang II (10^{-7}) +/- L-NAME (10^{-5}), Ang II +/- NGF (0.5 ng/ml) or Ang II + NGF + L-NAME were daily added to the cells. Vehicle-treated cells served as a negative control and cells treated with NGF (50 ng/ml, black column) served as a positive control. The data are representative of two independent experiments. Values represent means \pm SEM of n=20. ** p< 0.01 vs vehicle

Effect of NO on the differentiation in PC12W cells

Data from the previous set of experiments indicate that NO is involved in the Ang II-induced differentiation in PC12W cells, as inhibition of NO synthase by L-NAME abolished the effect of the peptide. In the following experimental setting, PC12W cells were exposed to various concentrations of freshly prepared sodium nitroprusside (SNP), an exogenous NO-donor, for 6 days, to investigate whether NO itself can stimulate the neurite outgrowth in these cells.

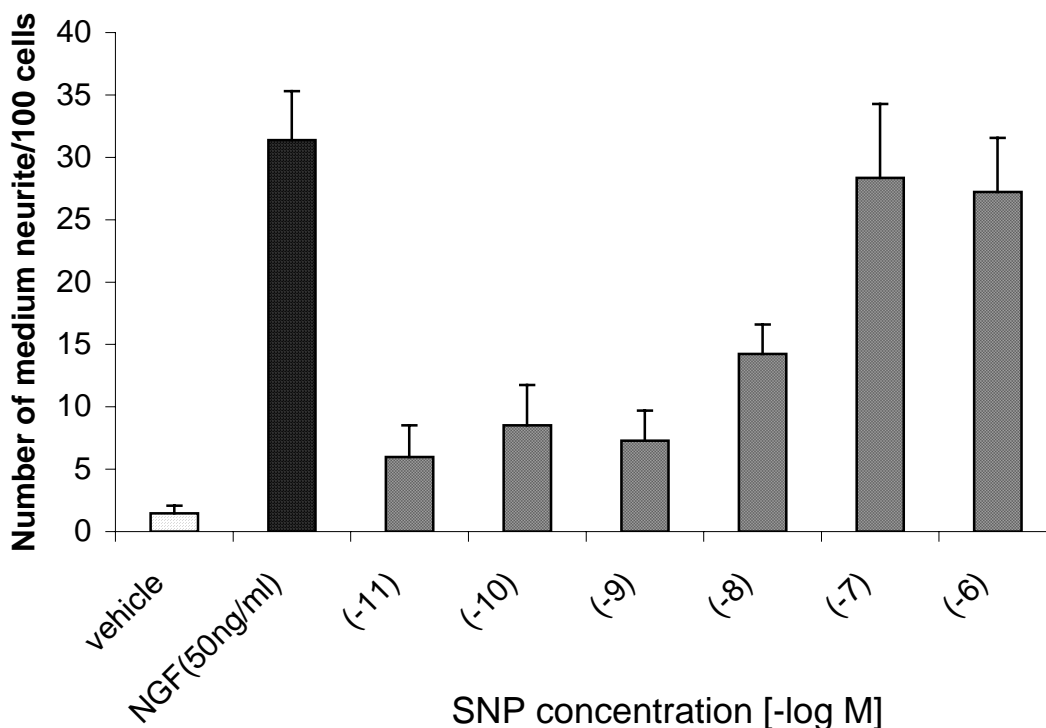


Fig 3-3. Effect of NO on the differentiation in PC12W cells. Quiescent PC12W cells were treated for 6 days with various concentrations of SNP (a NO-donor). SNP was added daily to the starve medium containing 0.5% iFCS. Negative control: cells treated with vehicle. Positive control: cells treated with NGF (50 ng/ml). The presented data is representative of two independent experiments. Values represent means \pm SEM of n=20.

The results show that exogenous NO induces neurite outgrowth in PC12W cells in a dose-dependent manner. Staining with trypan blue of PC12W cells exposed to SNP, revealed that SNP treatment at used concentration did not exert any toxic effects (data not shown).

Effect of NO synthase inhibition on the AT2 receptor-induced differentiation in PC12W cells: reversal by NO-donor

To further investigate the importance of NO in the AT2 receptor-induced differentiation, an identical protocol to that employed in the previous experimental setting was used. Outgrown neurites in PC12W cells stimulated with Ang II in the presence or absence of the NO-donor, sodium nitroprusside, when NO synthase was inhibited by L-NAME was quantified. To verify the reproducibility of the observed effects and to assure identical experimental conditions for all groups, which was necessary for an appropriate evaluation of the obtained results, treatment combinations already used in the last set of experiment were also included.

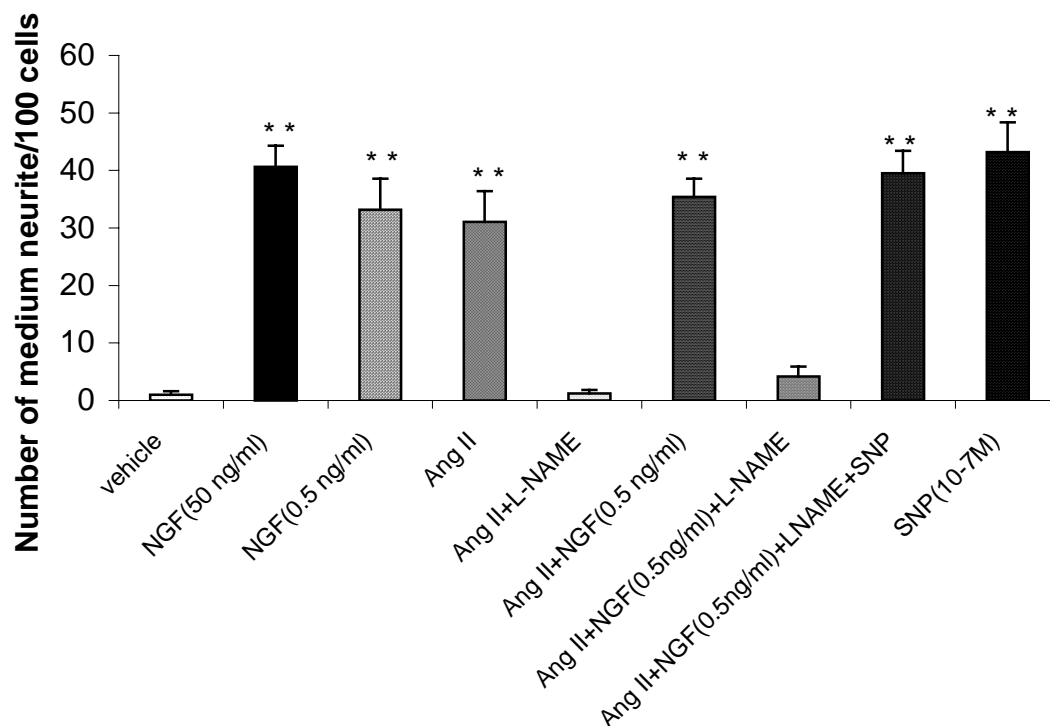


Fig 3-4. Effect of NO-donor on the differentiation in PC12W cells induced by Ang II after inhibition of NO synthase. Quiescent PC12W cells were daily stimulated with Ang II (10^{-7} M), Ang II + L-NAME (10^{-5} M), Ang II + NGF (0.5 ng/ml) + L-NAME and Ang II + NFG + L-NAME + SNP (10^{-7} M) for 6 days. Vehicle-treated cells represented the negative and cells treated with NGF (50 ng/ml) served as the positive control. The presented data are representative of two independent experiments. Values represent means \pm SEM of $n=20$. ** $p<0.01$ vs vehicle.

These results confirm the previous findings indicating the important role of NO in the Ang II-induced differentiation of PC12W cells. Inhibition of NO synthase abolished the effect of Ang II on neurite outgrowth, an effect which was reversed by the addition of an exogenous NO-donor.

cGMP and cell differentiation

Ang II-induced differentiation in PC12W cells is mediated by NO. NO activates a soluble guanylate cyclase to produce cGMP. cGMP might be a downstream mediator which activates intracellular events responsible for the initiation of differentiation. To test the hypothesis that cGMP can promote the cell differentiation, quiescent PC12W cells were treated with 8-bromo cGMP, a cGMP analogue which penetrates through the cell membrane. Again, the outgrowth of neurites was quantified.

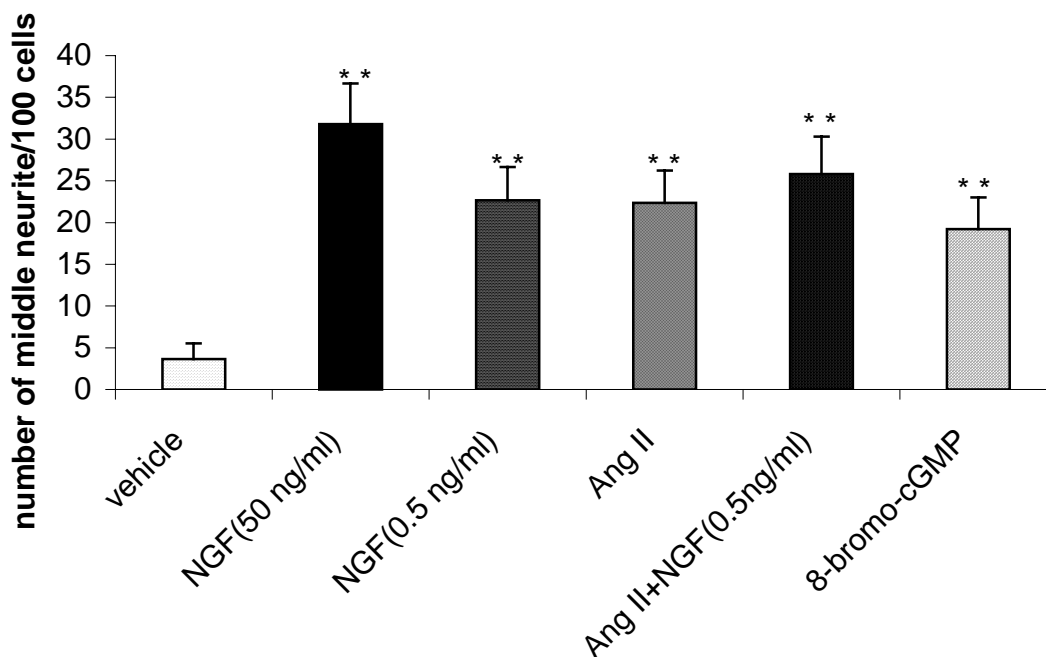


Fig 3-5. Effect of 8-bromo-cGMP on the differentiation in PC12W cells. Quiescent PC12W cells were daily treated with of Ang II (10^{-7} M) + /- NGF (0.5 ng/ml), 8-Bromo-cGMP (10^{-4} M) for 6 days. Vehicle-treated cells served as a negative control; cells treated with NGF (50 ng/ml) served as a positive control. The presented data are representative of two independent experiments. Values represent means \pm SEM of n=20. ** p<0.01 vs vehicle.

Fig 3-5 shows that the cGMP analogue, 8-bromo-cGMP, induced differentiation in PC12W cells, and that the neurite outgrowth-promoting effect of 8-bromo-cGMP at the concentration used was comparable to that of NGF or Ang II.

Ang II-induced differentiation in PC12W cells: Involvement of bradykinin.

Icatibant (HOE 140), the antagonist for the B2 receptor, has been shown to inhibit the AT2 receptor-mediated generation of cGMP in rat aorta homogenates (Gohlke et al., 1998). These findings point to a link between the AT2 receptor-mediated generation of cGMP and the B2 receptor. To study whether the B2 receptor is involved in Ang II-induced cell differentiation, PC12W cells were cultured with Ang II (10^{-7} M) in the presence or absence of icatibant (10^{-5} M) for 6 days and the outgrown neurites were quantified.

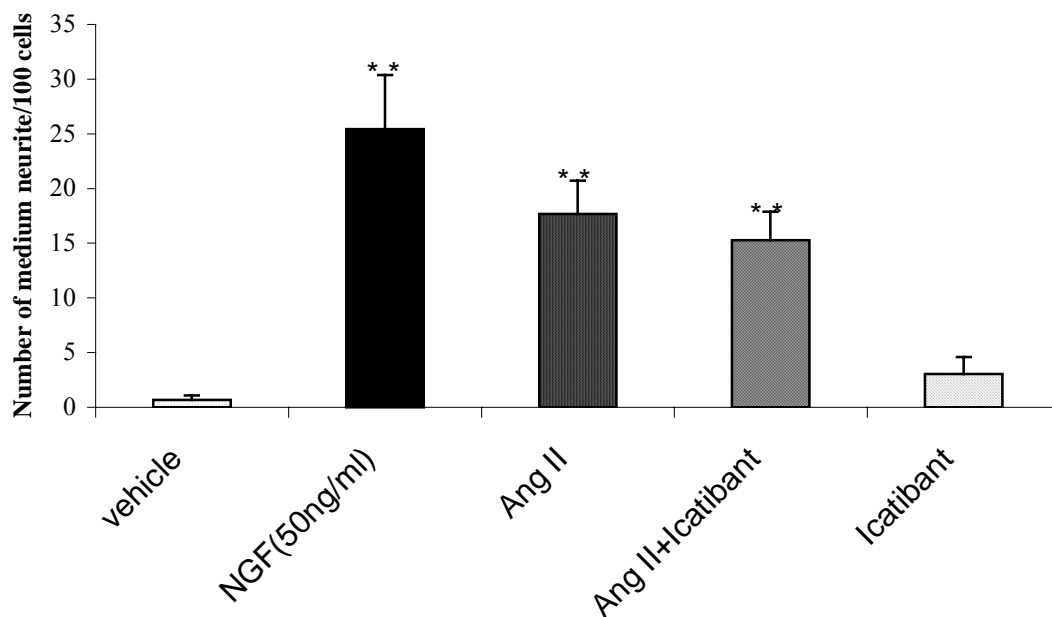


Fig 3-6. The effect of B2 receptor antagonist, icatibant on the Ang II-induced differentiation in PC12W cells. Quiescent PC12W cells were daily treated with Ang II (10^{-7} M) in the presence or absence of B2 receptor antagonist icatibant (10^{-5} M) for 6 days. Negative control: vehicle treated cells; positive control: cells treated with NGF (50 ng/ml). The presented data are representative of two independent experiments. Values represent means \pm SEM of $n=20$. ** $p < 0.01$ vs vehicle.

Fig 3-6 demonstrates that the B2 receptor antagonist did not inhibit the effect of Ang II on neurite outgrowth in PC12W cells. Present data indicate that the B2 receptor

was not a part of cellular and/or molecular mechanisms initiating cell differentiation in response to the AT2 receptor activation in PC12W cells.

Effect of Ang II on NO synthase mRNA level in PC12W cells

Ang II acting on AT2 receptors increases NO generation in PC12W cells. In the present study it is reported that BK, which represents one of the most potent activators of NO production, does not contribute to the AT2 receptor-mediated differentiation in P12W cells. In the last sets of experiments, it has been investigated, whether Ang II can directly modify the expression of NO synthase(s) to increase the NO generation in PC12W cells. It was attempted to answer the question whether activation of AT2 receptors could affect the expression of NO synthase(s) mRNA levels in PC12W cells and which NO synthase isoform is regulated by the AT2 receptor in these cells. mRNA levels of three different isoforms of NO synthase were examined using RT-PCR. Briefly, 2.5×10^4 /ml PC12W cells were planted in 10 cm dishes pre-treated with collagen A and incubated in normal medium for 24 hrs. The normal medium was replaced by starved medium to obtain quiescent PC12W cells. These cells were then stimulated with Ang II (10^{-7} M) and total RNA was isolated with Trizol-reagent 2, 4, 8, 10 and 12 hrs post stimulation with the peptide.

Fig 3-7 shows that Ang II up-regulated mRNA levels of endothelial NOS (eNOS) time- and dose- dependently with respect to post-stimulation time periods. The highest expression of eNOS mRNA was detected after 8 hrs (Ang II 10^{-7} M) and no further increase was detected after 8 hrs at longer time points.

Increased mRNA levels of neuronal NOS (nNOS) were observed in PC12W cells after treatment with Ang II (10^{-7} M), Although the nNOS mRNA level in PC12W cells was lower 8 hrs post-treatment with Ang II (10^{-7} M) compared to that of 2 hrs treatment, an exaggerated expression of the mRNA was detected 12 hrs post-stimulation with the peptide.

Surprisingly, inducible NOS (iNOS) mRNA could be reduced in PC12W cells after 2 hrs incubation with Ang II (10^{-7} M), but up-regulated at higher dose of Ang II (10^{-5} M) 8 hrs post stimulation with the peptide.

Taken together, three isoforms of NO synthase(s) were found to be expressed in PC12W cells. The levels of RNA of both constitutive NOS, neuronal NOS and endothelial NOS, were up-regulated following treatment with Ang II. However the

RNA levels of inducible NOS were at first reduced following stimulation with Ang II. The combined effects of NOS isoforms on Ang II induced differentiation in PC12W cells are still unknown.

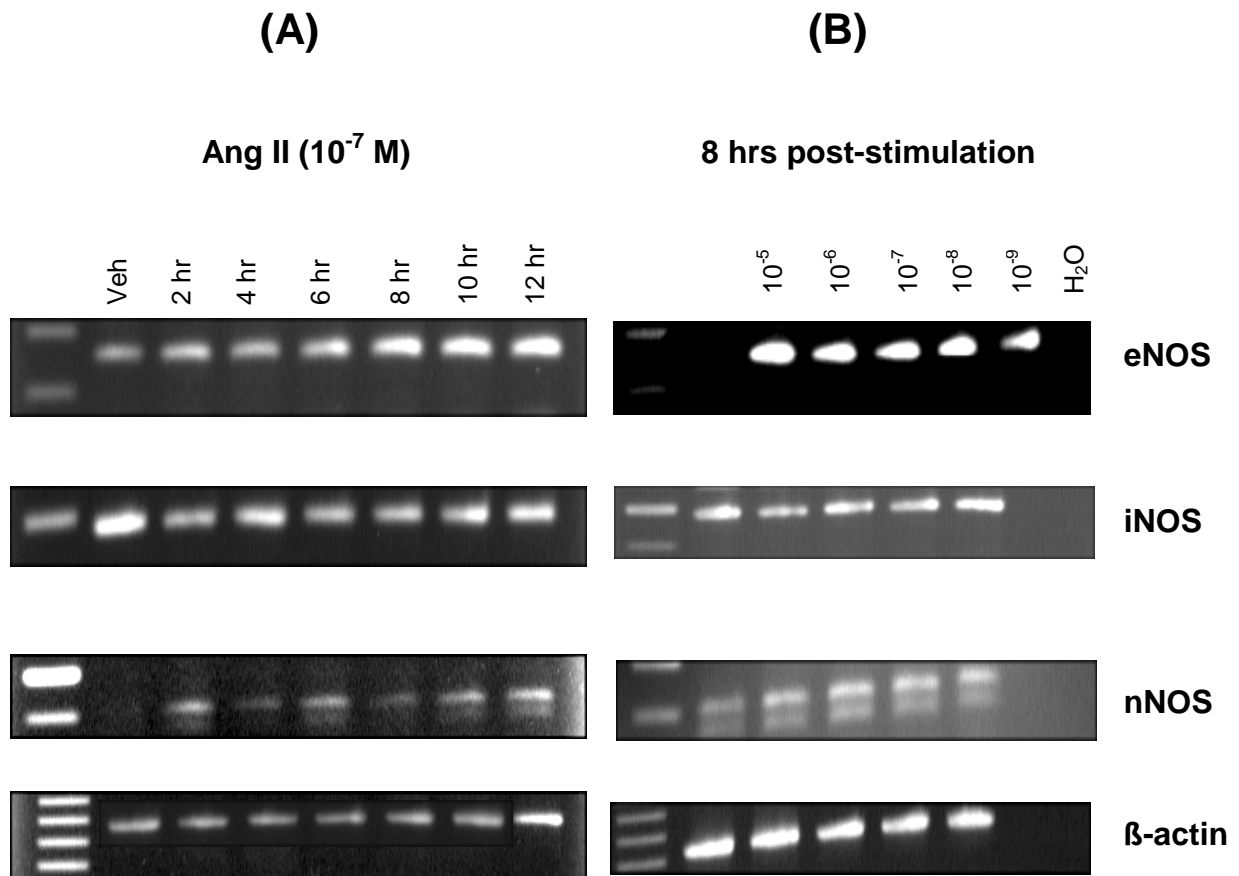


Fig 3-7. Expression of NO synthase(s) mRNAs in PC12W cells after stimulation with Ang II. Quiescent cells were incubated with Ang II (10^{-7} M) and total RNA was isolated 2, 4, 6, 8, 10 and 12 hrs post stimulation with the peptide. RT-PCR was carried out with 5 μ g of total RNA using specific eNOS, iNOS and nNOS primers in the PCR procedure. 10 μ l of each PCR product were loaded on 1.2 % agarose and run at 70 V for 90 minute. Bands were visualised by 0.5 μ g/ml of ethidium bromide. eNOS, iNOS and nNOS were identified by 384 bp, 581 bp or 534 bp bands respectively. (A) time- dependent regulation of NO synthase by Ang II (10^{-7} M) in PC12W cells. (B) dose- dependent regulation of NO synthase by Ang II (8 hrs after stimulation) in PC12W cells.

Evaluation of the angiotensin receptor subtypes in PC12W cells

It has been reported that PC12W cells express only the AT2 receptor at the passages lower than 18. To further confirm that the data obtained above are AT2 receptor-mediated, RT-PCR and binding assay were used to evaluate the angiotensin receptor subtype in PC12W cells. Cells were cultured in 10 cm dishes or 24 well plates with normal medium till confluence. Total RNA was isolated using Trizol reagent for the AT1 and AT2 receptor PCR. Fig 3-8 (A) shows that at the cell passages we used for the experiments there were only the AT2 receptor but not the AT1 receptor expression. Binding assay further confirmed the results (Fig.3-8).

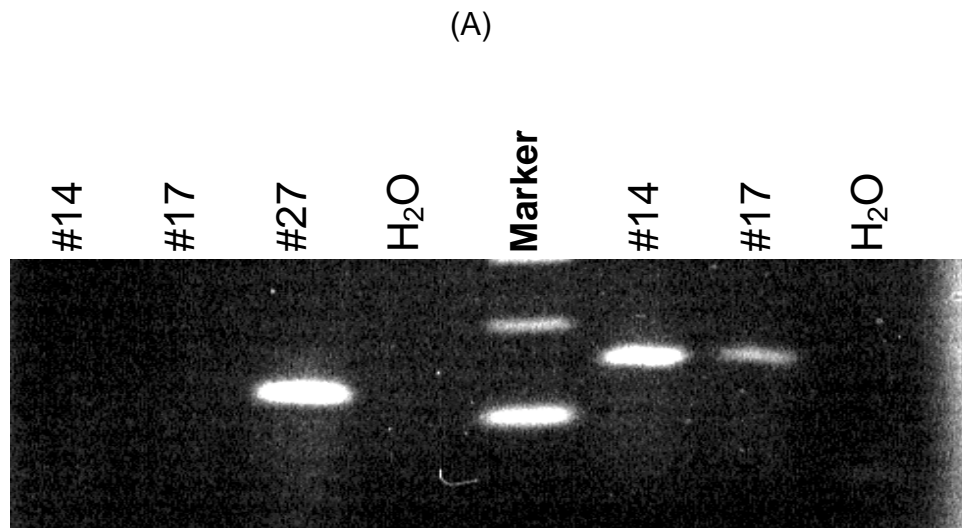


Fig 3-8. See following page for accompanying legend. Number represents the cell passages.

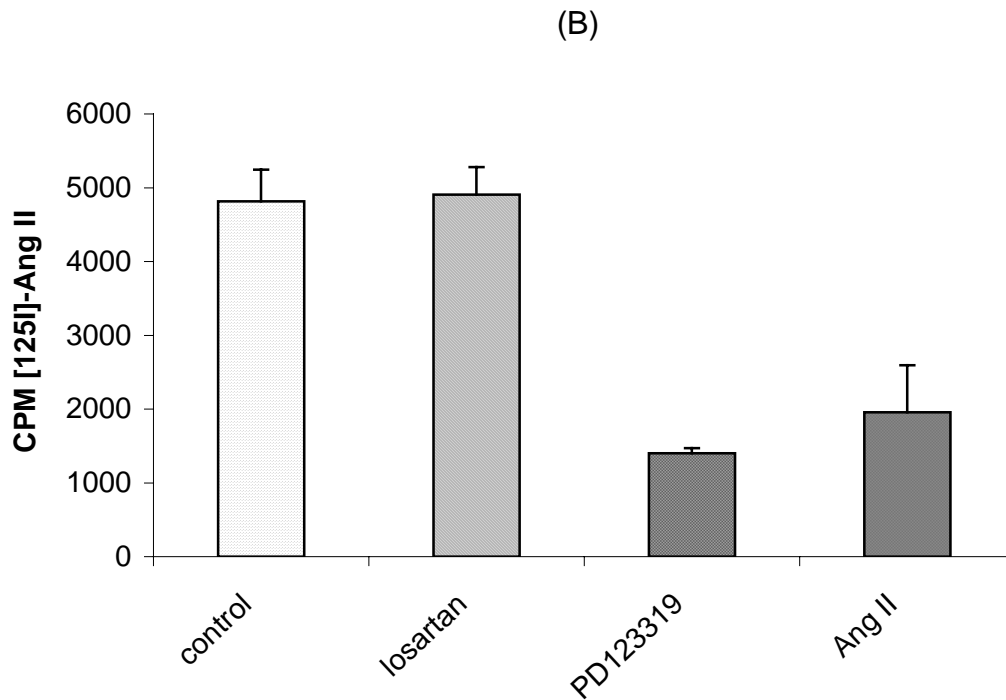


Fig. 3-8. AT1 and AT2 receptor assay for PC12W cells. (A). Total RNA was isolated from PC12W cells in different passages. 5 μ g of total RNA were used for RT-PCR. 15 μ l of PCR product were loaded on a 2.0% agarose gel and run at 70V for 90 min. AT1 and AT2 receptors were identified by 122 bp or 154 bp bands respectively. (B). [125 I]-Ang II binding activity in the presence of angiotensin receptor ligands on PC12W cells. Confluent PC12W cells were incubated with [125 I]-Ang II (40 pM/well) in the presence of Ang II, or PD123319, or losartan (1 μ M each) for 2 hrs, at 37°C. Data are shown of a single experiment. Data points correspond to an average of n =4.

Discussion (section I)

Expression of Ang II receptors in SK-UT-1 cells

A number of studies have reported that Ang II via its AT1 receptors can initiate proliferation, hypertrophy or hyperplasia in various tissues. In contrast, stimulation of AT2 receptors leads to anti-proliferation and promotes differentiation and may, under certain conditions, initiate cellular mechanisms leading to apoptosis (Unger, 1999). Both subtypes of Ang II receptors are also present in smooth muscle cells (SMC) of the uterus. In non-pregnant uterus, the AT2 receptors predominate and their expression varies dramatically during the menstrual cycle and are down regulated during gestation. AT1 receptors are also expressed in the myometrium and their numbers do not change during the menstrual cycle or gestation (Mancina et al., 1996).

The fact that Ang II acts, on one side, as a growth hormone promoting hypertrophy and hyperplasia and, on the other side, initiates differentiation and /or apoptosis, indicates that the peptide and its receptors may also play a role in processes related to the tumorigenesis. For instance, the AT2 receptor mRNA levels have been reported to be dramatically decreased in adrenal adenomas of both, Cushing's and Conn' syndroms (Kitamura et al., 1998).

SK-UT-1 cells represent a human uterus leiomyosarcoma cell line. No data were available whether SK-UT-1 cells express angiotensin receptors. Binding assays using whole cells from SK-UT-1 cells failed to reveal angiotensin binding sites. Similarly, no measurable amounts of mRNA for the AT2 receptor have been detected using PCR with standard condition, indicating that, if AT2 receptors are expressed in these cells, their number must be very low. Single cell PCR was successfully used in our laboratory to detect AT2 receptor mRNA in a single cardiomyocyte cells (Busche et al., 2000). Using this slightly modified method, we have been able to detect mRNAs for the AT2 receptor subtype. The mRNA levels were very low but clearly detectable indicating that these cells in fact express AT1 and AT2 receptors. The expression of the AT2 receptor was extremely low during the incubation with growth factors but considerably increased when the cells were serum deprived. Studies using other cell

lines or primary cells including myocytes isolated from neonatal rat heart, PC12W or R3T3 cells, have also reported that serum deprivation causes up-regulation of the AT2 receptor (Kijima et al., 1996; Li et al., 1998). The results from the present studies support the hypothesis that Ang II can contribute to the regulation of processes linked to tumorigenesis. When AT1 receptors are increasingly expressed, Ang II would exert growth-promoting actions and stimulate proliferation. On the contrary, activation of AT2 receptors would suppress growth and rather promote differentiation. Furthermore, the findings demonstrating the existence of the AT1 and the AT2 receptor in human uterus leiomyosarcoma cells and the regulatory effects of growth factors on the expression of the AT2 receptor, indicate that these cells may represent a relevant model to investigate the role of the angiotensin receptors in tumorigenesis.

Effect of Ang II on the proliferation of SK-UT-1 cells

The ³H-thymidine incorporation test provides a suitable method to investigate the rate of DNA synthesis and enable to draw conclusions on cell proliferation. Increased ³H-thymidin incorporation indicates enhanced mitogenic activity.

In our experiments carried out in promoting or quiescent SK-UT-1 cells, Ang II had no effect either on the cell numbers or on the rate of DNA synthesis. Furthermore, neither inhibition of AT1 receptors nor inhibition of AT2 receptors had any effect on proliferation of SK-UT-1 cells. Additional experiments in confluent cells (high density of cells in the culture), with supposedly up-regulated cell surface AT2 receptors, have also demonstrated that Ang II does not influence proliferation of these cells, nor does it interfere with mitogenic effects induced by growth factors. These data suggest that growth factors seem to represent a very strong mitogenic stimulus for these cells which cannot be affected by angiotensin receptors.

The failure of Ang II to reduce through activation of the AT2 receptor the ³H-thymidine incorporation in SK-UT-1 cells is rather surprising. Growth inhibitory effects of Ang II have been convincingly demonstrated in PC12W, and R3T3 cells, which express exclusively AT2 receptors, or in CEC, when AT1 receptors were inhibited by AT1 receptor antagonists. In all these cell lines, inhibition of ³H-thymidine incorporation is observed when the cells are exposed to Ang II. One explanation for

the failure of Ang II to inhibit proliferation of SK-UT-1 cells via AT2 receptors might be the fact, that in SK-UT-1 cells, which represent a very malignant tumor cell line, the mitogenic effects of growth factors are extremely exaggerated and they cannot be offset by a concomitant activation of AT2 receptors. Moreover, the low expression of AT2 receptors must also be considered as the overnight exposure of cells to growth factors (10% iFCS or 1% ITS) might down-regulate the expression of the AT2 receptors. We indeed observed that the expression of AT2 receptors was extremely low in proliferating compared to quiescent SK-UT-1 cells. Under these conditions the AT2 receptor-mediated anti-mitogenic effect could hardly be achieved. However, even at the absence of growth factors in the culture medium, the cell proliferation was not be influenced by the treatment with Ang II although higher AT2 receptor mRNA levels were detected. Rapid inactivation of Ang II or the antagonists in the medium can be excluded since these compounds retained full biological activity under similar culture condition in the experiment carried out in PC12W, R3T3 cells and CEC. In these experiments, Ang II acting on the AT2 receptor clearly attenuated proliferation and promoted differentiation.

The reason why the stimulation of AT2 receptors in SK-UT-1 cells did not induce any proliferation-inhibitory effects is presently unknown. It could be due to the fact that the AT2 receptor may exert its growth-inhibiting actions on various levels including modulation of the signaling of other, most probably, growth factor receptors or the activity of transcription factors. It can be assumed that these rapid proliferating tumor cells lack of the target molecule(s) mediating the growth-arrest following activation of AT2 signaling pathways, or that these target molecules are not expressed in sufficient amounts to inhibit proliferation.

Selective activation of AT1 receptors in SK-UT-1 cells unexpectedly failed to stimulate proliferation. Again, the link between the AT1 receptor signaling and downstream events promoting proliferation in cells with a rapid cell cycle may be absent or differ from those operating in other, slowly proliferating tumor cells, such as R3T3 and PC12W cells. Further experiments using in SK-UT-1 cells or other established cell culture models expressing AT1 and AT2 receptors might help to explain the discrepancies between the above mentioned findings and the present results.

Ang II and the expression of the differentiation markers, SM22 and calponin

Activation of AT₂ receptors in SK-UT-1 did not exert any growth-inhibitory actions. In further experiments, it was investigated whether activation of this angiotensin receptor subtype would induce differentiation.

Generally, to study cell differentiation, an analysis of morphological changes and/or a detection of specific differentiation markers can be employed. Several specific differentiation markers have been reported to evaluate the differentiation in SMC, such as α -SM actin (Blank et al., 1992), smooth muscle myosin heavy chain (Madsen et al., 1997), β tropomyosins (Kashiwada et al., 1997), calponin (Gimona et al., 1992; Shanahan et al., 1993; Duband et al., 1993), SM22 α (Solway et al., 1995), h-caldesmon (Yano et al., 1995) and α 1 integrin (Obata et al., 1997).

This study demonstrates that Ang II considerably increased SM22 mRNA expression in the presence of the growth factor, ITS. This effect was mediated by the AT₂ receptor, since firstly, it could be detected only when the AT₁ receptor was blocked by losartan and, secondly, pretreatment with the selective AT₂ receptor antagonist completely blocked the Ang II induced up-regulation of SM22 mRNA. Interestingly, the up-regulation of SM22 mRNA level was detected at the early time point, 1 hour post Ang II treatment, and no more detectable 3 hours post Ang II treatment.

SM22 is a 22-kDa SMC lineage-restricted protein associated with cytoskeletal actin filament bundles in contractile SMC thus representing an important differentiation marker for SMC (Duband et al., 1993; Lees-Miller et al., 1987). In VSMC, SM22 is expressed abundantly and exclusively during postnatal development. The expression of SM22 was also observed in cardiac and skeletal muscle cells, and it appears in the early embryogenesis about day E9 which suggests that the expression of the SM22 is associated with the early development of SMC.

The early induction of SM22 mRNA expression by the AT₂ receptor stimulation could be detected because the basal SM22 mRNA levels were low. A low expression of SM22 was associated with cellular events linked with the dedifferentiation of SMC

(Hayashi et al., 1998). The appearance of SM22 mRNA expression in SK-UT-1 cells may serve as a marker for the switch to cellular programs associated with differentiation. The up-regulation of SM22 mRNA levels by the AT2 receptor activation suggests that Ang II acting on the AT2 receptor can be considered as a factor promoting differentiation in uterus leiomyosarcoma cells by changing the phenotype of these tumor cells as a result of the cytoskeleton reorganization.

Three and 6 hours lasting stimulation of SK-UT-1 cells with Ang II in the presence or absence of angiotensin receptor antagonists and under the same culture conditions, an up-regulation of SM22 mRNA levels was observed in all experimental groups. The up-regulation of SM22 expression in all experimental groups after 3 and 6 hours of incubation can be related to the action of ITS. It has been reported that insulin and insulin-like growth factors (IGF-I and IGF-II) markedly prolong the differentiated phenotype in primary SMC (Hayashi et al., 1998). Even though there is not any data indicating that insulin regulates the expression of SM22 in SMC, it can be concluded that insulin induced the up-regulation of SM22 mRNA observed at the later time points. It is assumed that, after 3 or 6 hours of stimulation of SK-UT-1 cells with medium containing 1% ITS (insulin), any induction of SM22 mRNA levels by AT2 receptor activation was overridden by their up-regulation caused by insulin.

It has been reported that the AT2 receptor mRNA expression in the mouse aorta is low during the early fetal development (E13-E15) but increases rapidly during later phases of the fetal development (E18-E21). In parallel with the increased AT2 receptor occurrence, the mRNA expression of calponin in the aorta tissue is “switched on” at E13-E15 and continues to increase thereafter. Thus, the rate of calponin synthesis is closely associated with the AT2 receptor expression which suggests the calponin may be regulated by the AT2 receptor (Yamada et al., 1999).

It has been well documented that calponin is expressed in differentiated SMC but in dedifferentiated cells, calponin expression is down regulated. The present results show that Ang II can up-regulate calponin in SK-UT-1 cells. A continuous exposure to the peptide for 1 and 3 hours caused an up-regulation of calponin mRNA when the AT1 receptor was blocked by losartan. The effect of Ang II was abolished after 6 hours of incubation. Correspondingly, increased levels of calponin protein were

detected after 9 but not any more after 12 hours of incubation with Ang II plus losartan (see Fig 1-9).

The expression of SMC differentiation genes seems to depend on the cell type and the cell phenotype. In differentiated leiomyomas cells, calponin expression cannot be further stimulated because the basal expression of calponin is relatively high, but it can be dramatically up-regulated in differentiated leiomyosarcoma cells in which the basal expression is low. These findings indicate that the regulation of calponin rather occurs in malignant tumor cells compared to benign tumor cells. Correspondingly, the AT2 receptor-mediated up-regulation of calponin expression was observed in the leiomyosarcoma cells with rapid cell cycle growth. AT2 receptors have been reported to be decreased in cells incubated with growth factors. Indeed, a very low expression of AT2 receptor mRNA levels in SK-UT-1 cells cultured 48 hours with ITS was observed (Fig 1-1). When Ang II and losartan were added to SK-UT-1 cells together with ITS, the peptide stimulated calponin expression up to 3 hours of the stimulation since AT2 receptors were still present. The failure of Ang II to stimulate calponin expression 6 hours after the onset of the incubation with the peptide was most probably due to a reduction of AT2 receptors induced by ITS.

Only few investigators have investigated the effect of the AT2 receptor on calponin gene regulation. The conclusions are rather equivocal. Dulin and co-workers (2001) reported that Ang II increased calponin levels in rat aorta SMC in a dose- and time-dependent manner. This Ang II effect was AT1 receptor-mediated since the AT1 receptor antagonist, losartan, abolished it, while the AT2 receptor antagonist, PD123319, was without effect. The authors used primary-derived aortic SMC from adult Wistar-Kyoto rats between passage 5 and 15. The cells were treated with Ang II in serum-starved medium, and the authors did not report on the numbers of AT1 and AT2 receptors in their cell preparation. Similar conclusions have been drawn from experiments carried out on rat aortic SMC *in vitro* (di Gioia et al., 2000) and *in vivo* (Castoldi et al., 2001). Furthermore, Ang II was reported to down-regulate the level of calponin in primary SMC isolated from the gizzard of 15-day old chick embryos while insulin had an opposite effect (Hayashi et al., 1998). Compared to Ang II, serum, EGF, TGF β s and PDGFs reduced the calponin mRNA expression to a negligible level and produced a marked de-differentiation. Again, the authors did not report on

the expression of AT1 and AT2 receptors. Taken together, it appears that both Ang II receptors contribute to the regulation of calponin expression and differentiation processes in SMC depending on the origin and type of the cells and experimental conditions.

PC12W cells treated with Ang II increasingly express biochemical differentiation markers and exhibit typical morphological changes, such as neurite outgrowth, which also represents a typical differentiation marker. It was expected that the up-regulation of two differentiation markers, SM22 and calponin, by Ang II in SK-UT-1 cells would also be associated with morphological changes of these cells. In fact, the cultured cells grew in an irregular fashion similar to the untreated controls. This may be due to the fact that the AT2 receptor-mediated up-regulation of differentiation markers in SK-UT-1 cells was investigated in proliferating cells while the Ang II-induced differentiation in PC12W cells was carried out in quiescent cells. As already mentioned above, calponin levels are also dramatically up-regulated in serum deprived quiescent SK-UT-1 cells. These non-proliferating cells with such a high calponin level do not respond with additional expression of calponin to compounds inducing differentiation. However, when these quiescent cells are exposed to ITS, a growth factor, Ang II still induces biochemical differentiation markers, but any attempts to induce morphological changes typical for the differentiation are obviously overridden by the further proliferation-promoting effects of ITS.

Effect of Ang II on the apoptosis of uterus leiomyosarcoma cells

Apoptosis plays a major role during development, homeostasis and immune response in multicellular organisms. It has been reported that Ang II induces apoptosis in many tissues and cell lines through AT2 receptor activation, for instance in PC12W cells (Gallinat et al., 1999; Yamada et al., 1996), fibroblasts (Li et al., 1998), R3T3 cells (Yamada et al., 1996), VSMC (Nagashima et al., 2001), cardiomyocyte (Goldenberg et al., 2001) and endothelial cells (Hamet & deBlois, 2001). The dramatic increase in calponin mRNA level in serum deprived SK-UT-1 cells and the present observation of an AT2 receptor-mediated up-regulation of calponin mRNA and protein levels raised the speculation that the AT2 receptor activation in these cells may induce apoptosis. Indeed, Ang II caused apoptosis in

SK-UT-1 cells after 36 or 48 hours of peptide treatment. This effect was AT2 receptor-mediated since it was observed only when AT1 receptors were blocked. PD123177 treatment abolished the apoptosis-promoting effect of Ang II in these cells. The reason why Ang II induced apoptosis in SK-UT-1 only at time points 36 or 48 hours is at the present unknown.

Low expression of AT2 receptors in these cells treated with culture medium containing 1% ITS might be responsible for the delayed apoptotic effect of Ang II. Following serum deprivation, the cells became quiescent and the number of AT2 receptors was relatively high. Then the cells were incubated with medium containing ITS which, on one side, promotes proliferation and on the other side down-regulates the AT2 receptor expression. During the first 6 - 9 hours, when the number of AT2 receptors was still high, Ang II enhanced the expression of differentiation markers. This differentiation-promoting action of Ang II was directly opposed to the proliferation-promoting action of ITS. At later time points, ITS suppressed the expression of AT2 receptors, their numbers were steadily decreasing and Ang II was not able to induce differentiation markers. Since proliferation is preferred in SK-UT-1 cells incubated with a growth factor, Ang II acting on AT2 receptors promoted apoptosis only in a small population of these cells. Ang II acting on AT2 receptors can promote both, tumor cell differentiation and apoptosis, depending on a number of factors such as the presence of growth hormones, number of membrane AT2 receptors and the ratio of AT1/AT2 receptors. The tendency for malignant cells to undergo apoptosis is directly related to tumor cell differentiation (Saegusa et al., 1995), and it cannot be excluded that the up-regulated calponin is one of the predictors of apoptosis since enhanced expression of this protein preceded the induction of apoptosis in SK-UT-1 cells.

Discussion (section II)

Effect of Ang II on the Zfhep expression in CEC

As already mentioned, the control of cellular proliferation, differentiation and apoptosis appears to belong to the major actions of Ang II. Growth stimulatory effects induced by Ang II are usually associated with the stimulation of AT1 receptors (Aceto

& Baker, 1990; Paquet et al., 1990), while AT2 receptors have been shown to inhibit the proliferation of various cell types *in vitro* (Meffert et al., 1996; Stoll et al., 1995; Tsuzuki et al., 1996) and *in vivo* (Munzenmaier & Greene, 1996; Nakajima et al., 1995). While the signaling cascade of the AT1 receptor is well characterized, the signaling of the AT2 receptor remains still a matter of controversy. A hallmark of AT2 signaling appears to be the activation of phosphatases which inhibit the phosphorylation steps in growth-promoting signaling. Even less is known about target genes of the AT2 signaling cascade. To further characterize downstream events of AT2 signaling and the AT2-induced antigrowth actions, differentially expressed genes following stimulation of AT2 receptors in PC12W cells was identified using differential mRNA display. *Zfhep* is one of the three genes which were successfully confirmed by Northern Blot and which seem to be directly related to the proposed function of the AT2 receptor. *Zfhep* belongs to the *Zfhep* family of Drosophila genes that are required for differentiation of the CNS and in the regulation of cell fate (Lai et al., 1991; Lai et al., 1993). The AT2 receptor is known to induce differentiation of PC12W cells (Meffert et al., 1996). Previous findings have demonstrated that in quiescent PC12W cells Ang II transiently induced the expression of *Zfhep*, one and three hours following stimulation, an effect that was AT2-specific since it was abolished by the AT2 antagonist, PD123177. The present study was designed to determine whether the observed induction of *Zfhep* mRNA was specific to cells of neuronal origin, which undergo differentiation, or whether *Zfhep* mRNA induction can also be observed in cells of vascular origin; i.e., CEC. This might be of a particular interest because, while PC12W cells express only AT2 receptors, CEC express both angiotensin receptor subtypes. Correspondingly, these cells respond differently to Ang II depending on which receptor subtype predominate.

The present results clearly demonstrate that the AT2 receptor also induced *Zfhep* in CEC. However, the effect of Ang II in CEC was only detectable when AT1 receptors were blocked by losartan. The finding, that simultaneous stimulation of both receptors by Ang II was ineffective on *Zfhep* expression suggests that the AT1 receptor does not stimulate *Zfhep* transcription but down-regulates the AT2-receptor-induced *Zfhep* mRNA expression. This is an interesting finding, because in all previous studies, the Ang II- mediated induction of transcription factors was attributed to the AT1 receptor, and this effect was unopposed by the AT2 receptor.

This is the first report which describes activation of a transcription factor via AT2 receptor activation which is opposed by the AT1 receptor. Furthermore, the results presented also show that the induction of *Zfhep* mRNA is not specific to cells of neuronal origin which undergo differentiation, but can also be observed in cells of vascular origin. In addition, *Zfhep* not only represents a suitable marker for AT2 receptor activation but may also link AT2 signaling and downstream events involved in the proposed function of the AT2 receptor in processes occurring during development and regeneration.

From these results, it is apparent that *Zfhep* can represent a suitable marker for the study of the AT2 receptor activation and subsequent elucidation of signaling events involved in the regulation of cell growth and differentiation. For example, a yeast-2-hybrid screen revealed an interaction between the third intracellular loop of the AT2 receptor and a member of the EGF receptor family, ErbB3, as a part of a novel signaling mechanism for the AT2 receptor mediated inhibition of cell growth (Knowle et al., 2000). Our initial experiments for the differential mRNA display examined the influence of the AT2 receptor activation upon EGF-induced growth in PC12W cells and led to the discovery of *Zfhep*. Therefore, *Zfhep* may represent an important link between early events in receptor-receptor cross talk, such as AT2-ErbB3, and downstream target genes involved in cellular growth and differentiation. The presence of a homeobox domain implies *Zfhep* in putative actions on differential genes. The mouse homologue gene, ZEB, was shown to down-regulate the activity of a number of transcription factors involved in cell differentiation, such as c-myc, NF- κ B p65, ITF-1 and myoD (Postigo & Dean, 2000). Since differentiation processes require positive as well as negative regulation of transcription factors to ensure a proper temporo-spatial pattern of gene expression, it has been postulated that ZEB is involved in the orchestration of differentiation events. Similarly, the AT2 receptor has been proposed to be involved in events associated with differentiation. It is conceivable that via the induction of *Zfhep*, the AT2 receptor can exert its role in the orchestration of differentiation during embryogenesis.

Discussion (section III)

Ang II-induced differentiation in PC12W cells: The role of the AT2 receptor

In the last part of this study, the signaling pathways associated with the AT2 receptor activation in PC12W cells, which express almost exclusively the AT2 receptor at low cell passages (< 18), were investigated. Results obtained previously in our laboratory have shown that Ang II induces neurite outgrowth in these cells. The outgrowth of neurites represents a fundamental morphological process associated with neuronal differentiation, development and remodeling in neuronal tissue. The present findings demonstrate that generation of nitric oxide resulting in an increased formation of cGMP constitutes, at least in part, the mechanism, by which Ang II promotes the differentiation of PC12 W cells. The present results also indicate that Ang II, via the AT2 receptor, time- dependently up-regulates the transcription of eNOS and nNOS.

Both, axons and dendrites sprouting from cells in vitro are named neurites, and are fundamental morphological characteristics for neuronal differentiation. In the present experiments, neurite sprouting was used as a marker to investigate the role of bradykinin and NO in the Ang II-induced differentiation of PC12W cells.

The expression of mRNAs coding for the angiotensin receptors was examined by RT-PCR at different cell passages. At cell passages less than 17, AT1 receptor mRNA was not detected. Angiotensin receptor binding assays also confirmed that PC12W cells used for the present studies expressed only the AT2 receptor.

Several studies have demonstrated that the differentiation of NG108-15 cells initiated by addition of cAMP, DMSO or by serum deprivation selectively increased AT2 receptor expression, but there was no obvious correlation between the extent of AT2 receptor expression and the differentiation process (Laflamme et al., 1996; Seidman et al., 1996). In the present study, it was also found that the expression of the AT2 receptor was increased after serum deprivation but not affected after incubation with Ang II or NGF for 6 days (data not shown). In line with previous findings, AT2

receptor mRNA levels did not correlate with morphological changes related to differentiation.

PC12W cells stimulated with NGF and NG108-15 cells treated with cAMP analogues are two models frequently used to study neuronal differentiation (Laflamme et al., 1996; Tojima et al., 2000). Recent studies have demonstrated that Ang II by activating the AT2 receptor promotes differentiation in both neuronal cell lines as well as in microexplant cultures of rat cerebellum (Cote et al., 1999; Laflamme et al., 1996; Meffert et al., 1996). The signal transduction mechanisms of these AT2 receptor-mediated effects are still a matter of controversy. An NO-induced neuronal differentiation was firstly described in PC12 cells in which NGF increased NOS expression (Peunova & Enikolopov, 1995). Recently, NO has been reported to act as a second messenger directly involved in the action of Ang II on neuronal differentiation in NG108-15 cells (Cote et al., 1998). Although both, NGF and Ang II can increase NO production, the downstream signaling events activated by each of these compound are quite different. In NG108-15 cells, the AT2 receptor-mediated neurite outgrowth was accompanied by a decrease in P21^{ras} activity and by a delayed increase in the activity of mitogen-activated protein kinases (MAPK), ERK1 (p44^{mapk}) and ERK2 (p42^{mapk}) (Gendron et al., 1999). On the other hand, in PC12W cells stimulated with NGF, induction of neurite outgrowth was initiated by an increase in P21^{ras} activity and by a rapid and sustained increase in p42/p44^{mapk} activity (Cowley et al., 1994; Pang et al., 1995). It appears, that in both models, the increase in p42/p44^{mapk} activity is a prerequisite for the neurite outgrowth induced by the AT2 receptor (Gendron et al., 1999; Stroth et al., 2000). In quiescent N1E-115 neuroblastoma cells expressing only the AT2 receptor, basal MAPK activity was not affected by Ang II treatment. However, Ang II prevented the fetal-calf serum as well as epidermal growth factor-induced activation of ERK1 and ERK2 (Bedecs et al., 1997). The Ang II-induced neurite outgrowth is also associated with alterations in the levels of microtubule-associated proteins (MAP). Ang II induces up-regulation of MAP2c in NG108-15 cells and MAP2 in PC12W cells, but down-regulates MAP1B in the latter (Laflamme et al., 1996, Stroth et al., 1998). Taken together, these studies point to a specific role of the AT2 receptor in neuronal cell differentiation and nerve regeneration via a cytoskeleton reorganization.

The effect of NO and cGMP on the AT2 receptor-mediated differentiation in neuronal cells

NO appears to represent the link between AT2 receptor stimulation and changes in MAPK's or MAP's associated with differentiation. In the present study, evidence is provided that NO is a mediator of the Ang II-induced cell differentiation in PC12W cells. Firstly, blockade of NO synthase by L-NAME blunted the effect of Ang II on differentiation in these cells. Secondly, application of sodium nitroprusside, an exogenous NO-donor, mimicked the morphological changes observed in PC12W cells after AT2 receptor activation. In accordance with these findings, the morphological differentiation of NG108-15 cells induced by Ang II was also mediated by NO (Cote et al., 1998). NO was also reported to represent a crucial factor in the mechanisms responsible for the NGF-induced neuronal differentiation of PC12W cells (Peunova & Enikolopov, 1995). In the latter study, NGF was shown to activate NO synthase resulting in increased generation of NO. NO seems to be an important factor mediating the switch to cytoostasis at the beginning of the differentiation events.

Cellular actions of NO can be mediated directly; e.g., by protein nitrosylation or by activation of soluble guanylate cyclase to produce cGMP. The present data suggest that the effects of NO are mediated by cGMP. Daily application of the cGMP agonist, 8-brom-cyclic GMP, was as efficient as Ang II in inducing the neurite outgrowth, indicating that activation of soluble guanylate cyclase by NO to increase the generation of cGMP most probably mediates the AT2 receptor-induced differentiation in PC12W cells. These observations are in line with those of Gendron et al (2002) who have reported that activation of the AT2 receptor in NG108-15 cells increases cGMP levels. We have also noticed, that Ang II dose-dependently stimulated the production of cGMP in PC12W cells. The Ang II-induced cGMP formation was rapid and transient and could be abolished by co-incubation with the AT2 receptor antagonist, PD123319. Similar to these findings, enhanced cGMP formation was also observed in neuroblastoma neuro-2A cells following Ang II treatment. The proposed mechanisms involve activation of the soluble guanylate cyclase by NO (Chaki & Inagami, 1993). Taken together, these results strongly suggest that the NO/cGMP pathway is involved in the AT2 receptor-mediated differentiation in neuronal cells.

However, several other, hitherto unknown factors and mechanisms may also play a role in the processes associated with the differentiation of cells of neuronal origin, especially, when Ang II or the AT2 receptor are not involved. Thus, Hindley et al (1997) also reported that NO enhanced the NGF-induced neurite outgrowth in PC12 cells by a cGMP dependent pathway, but in the absence of NGF, NO failed to elicit neurite outgrowth. Similar findings were reported by Phung et al (1999) who showed that sodium nitroprusside alone did not stimulate the outgrowth of neurites in PC12 cells. Furthermore, the differentiation-promoting effects of NO in the presence of NGF were independent of the guanylate cyclase/cGMP pathway (Phung et al, 1999).

Bradykinin and the differentiation induced by Ang II in PC12W cells

Our group has demonstrated in SHRSP that a rise in plasma Ang II concentration stimulated aortic cGMP generation by an AT2 receptor dependent mechanism. This effect of Ang II was abolished by blockade of the bradykinin B2 receptor as well as by NO-synthase inhibition suggesting that the Ang II-induced NO generation was mediated by bradykinin (Golhke et al., 1998). In the present study Ang II as well as bradykinin increased cGMP in PC12W cells. As expected, the enhanced cGMP production after treatment the cells with bradykinin could be completely abolished by the B2 receptor antagonist, icatibant. On the other hand, the Ang II-induced increase of cGMP levels was not reduced by icatibant, indicating that the generation of NO in response to Ang II in PC12 W cells was not mediated by bradykinin. In contrast to these findings, in bovine aortic endothelial cells, Ang II concentration-dependently increased both, the production of kinins as well as of cGMP (Korth et al., 1995; Wiemer et al., 1993,). The mechanism may involve activation of kininogenases by an AT2 receptor which would result in intracellular acidification and subsequent generation of bradykinin, as proposed in a recent study carried out in VSMC over-expressing the AT2 receptor (Tsutsumi et al., 1999). In all these studies, the effect of Ang II on cGMP production was completely abolished by bradykinin B2 receptor blockade indicating that bradykinin represents a crucial factor for the Ang II-induced NO production in VSMC or endothelial cells. These findings demonstrate that the cellular mechanisms promoting NO production in response to Ang II differ between cells of neuronal origin, such as the PC12 W cells, and endothelial or VSMC.

In the present experiments, B2 receptor blockade did not significantly block the effects of Ang II on the differentiation of PC12W cells. These results clearly demonstrate that the Ang II-induced NO generation in PC12W cells does not require the generation of bradykinin and rather results from a direct activation of NOS. Indeed, Ang II, via activation of AT2 receptors, time- and dose-dependently up-regulated the expression of eNOS in PC12W cells, but simultaneously reduced the expression of iNOS. The underlying mechanisms are not clear and further experiments are required to explain these findings.

In conclusion, the presented data strongly supports previous findings demonstrating that Ang II induces differentiation in PC12W cells by activation of AT2 receptors. The data also indicate that NO and cGMP, but not bradykinin, mediate the AT2 receptor induced neurite outgrowth, which is intimately associated with the regeneration and remodeling of neuronal tissue.

Summary

The major actions of Ang II, the main effector peptide of the renin-angiotensin system, are mediated by two G-protein coupled angiotensin receptor subtypes, the AT1 and the AT2 receptor. Most of the classical actions of Ang II in fluid and blood pressure homeostasis are mediated by the AT1 receptor, however, this receptor type may also initiate proliferation, hypertrophy and growth in various tissues. The AT2 receptor is expressed at high density during fetal development and is less abundant in adult tissues. Ang II via the AT2 receptor may act as modulator of biological programs involved in embryonic development, tissue regeneration and protection, differentiation and in the initiation of processes leading to programmed cell death. There is substantial evidence that the AT2 receptor can offset or counteract the effects mediated by the AT1 receptor on cell proliferation.

There is evidence indicating that Ang II interferes with a number of cellular processes in tumor cells. A useful tool in studying the role of angiotensin receptors in tumor tissue are leiomyosarcoma SK-UT-1 cells which express both angiotensin receptor subtypes. Experimental results obtained in these cells might be representative of AT1 and AT2 receptor functions in tumor cells. To explore the role of AT1 and AT2 receptors for the proliferation and growth of the human leiomyosarcoma, The expression of angiotensin receptors in leiomyosarcoma cell line SK-UT-1 and the effects of Ang II on the proliferation, differentiation and apoptosis in this cells line were studied. RT-PCR analysis revealed that SK-UT-1 cells express both subtypes of angiotensin receptors. Activation of neither receptor subtype did alter ³H-thymidine incorporation and the number of cells. However, stimulation of AT2 receptors up-regulated mRNA levels of smooth muscle cell differentiation markers, SM22 and calponin. An effect which was, however, not associated with morphological changes of these cells. This was due to the fact that the AT2 receptor-mediated up-regulation of differentiation markers in SK-UT-1 cells was investigated in cells cultured in the presence of the growth factor, insulin transferring selenium. Activation of the AT2 receptor in SK-UT-1 cells also induced apoptosis which was detectable 36 or 48 hours post receptor stimulation. These results can be interpreted as an effort of Ang

II acting on the AT2 receptor to antagonise proliferation of tumor cells, to promote their differentiation or to induce apoptosis.

Further experiments were designed to study the signal transduction pathways involved in the AT2 receptor-induced differentiation of coronary endothelial cells (CEC) and PC12W cells. In CEC, AT2 receptor stimulation induced *Zfhep* mRNA expression. *Zfhep* is protein directly involved in processes associated with the cell differentiation. *Zfhep* mRNA up-regulation could only be detected when the AT1 receptor was inhibited, demonstrating a negative-regulatory influence of AT1 receptors on AT2-receptor mediated actions. *Zfhep* not only represents a suitable marker for the AT2 receptor-induced differentiation of CEC, but it also links the AT2 receptor signaling and downstream events involved in the development and regeneration. The induction of *Zfhep* indicates that the AT2 receptor may play a role in the orchestration of intracellular events associated with embryonic development or differentiation.

Data presented in the third part of this thesis also demonstrate that Ang II increases in PC12 W cells the outgrowth of medium and large neurites, which represent typical morphological markers of neuronal differentiation. Evidence is provided that NO is the mediator for the Ang II-induced cell differentiation in PC12W cells since: i) Inhibition of NO synthase by L-NAME, a NO synthase inhibitor, blunted the effect of Ang II on the differentiation, and, ii) application of sodium nitroprusside, an exogenous NO-donor, mimicked the morphological changes induced by AT2 receptor activation in these cells. The present data strongly indicate that activation of guanylate cyclase resulting in enhanced formation cyclic GMP (cGMP) is also involved in the Ang II-induced differentiation of PC12W cells. Bradykinin, similarly to Ang II, increased cGMP content and this effect was completely abolished after pretreatment with the selective B2 receptor antagonist, icatibant.

These data demonstrate that Ang II, via activation of AT2 receptors, can stimulate cell differentiation in PC12W cells by NO-related and cGMP-dependent mechanisms. The results presented in this thesis identify some intracellular components of the signaling pathways activated by the AT2 receptor involved in the proposed function of this receptor in the development, regeneration and differentiation.

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