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# Angiotensin II-induced apoptosis in rat cardiomyocyte culture: a possible role of $AT_1$ and $AT_2$ receptors

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## Abstract

**Objectives—**To investigate the mechanism of angiotensin II-induced apoptosis in cultured cardiomyocytes by determining which receptor subtype is involved, and what is the relationship between intracellular $Ca^{2+}$ changes and apoptosis.

**Design and methods—**Neonatal rat cardiomyocytes were pretreated with either the $AT_1$ antagonist irbesartan or the $AT_2$ antagonist PD123319 before exposure to angiotensin II. Apoptotis was evaluated using morphological technique, staining nuclei by Feulgen and Hoechst methods followed by image analysis and by *in situ* terminal deoxynucleotidyl transferase nick-end (TUNEL) labelling. TUNEL-positive cardiocytes were distinguished from other cells by double staining with α-sarcomeric actin. Intracellular $Ca^{2+}$ changes were assessed by indo-1 fluorescence microscopy, and the effect of $Ca^{2+}$ on angiotensin II-induced apoptosis was tested using the calcium channel blocker verapamil.

**Results—**Exposure to angiotensin II (10 nmol/l) resulted in cell replication and a three-fold increase in programmed cell death ($P < 0.05$). Pretreatment with either irbesartan (an $AT_1$receptor antagonist, 100 nmol/l) or PD123319 (an $AT_2$ receptor antagonist, 1 μmol/l) prevented the angiotensin II-induced apoptosis, indicating the presence of both $AT_1$ and $AT_2$receptors on cardiomyocytes. Exposure of myocytes to angiotensin II caused an immediate and dose-dependent increase in the concentration of intracellular free $Ca^{2+}$ that lasted 40–60 s. The effect was sustained in a $Ca^{2+}$ free medium. Pretreatment of cells with irbesartan (100 nmol/l) and PD123319 (10 μmol/l) blocked $Ca^{2+}$ elevation. Pretreatment with verapamil (10 μmol/l) prevented angiotensin II-induced apoptosis.

**Conclusions—**Angiotensin II-induced apoptosis in rat cardiomyocytes is mediated through activation of both $AT_1$ and $AT_2$ receptors. The apoptotic mechanism is not related to the immediate angiotensin II-induced $Ca^{2+}$ rise from intracellular stores. However, it is accompanied by cardiomyocyte proliferation and requires $Ca^{2+}$ influx through L-type channel activity.

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**Keywords**

angiotensin II; $AT_1$ and $AT_2$ receptors; apoptosis; cardiomyocytes; $Ca^{2+}$ concentration

## Introduction

Programmed cell death (apoptosis) has been shown to occur during ageing and in various cardiac pathological processes [1–5]. Angiotensin II has been demonstrated to induce programmed cardiomyocyte death *in vitro* [6,7], and a direct correlation was found between angiotensin converting enzyme (ACE) activity and the apoptotic index in spontaneous hypertensive rats [8]. Nevertheless, the ability of angiotensin II to produce apoptosis is controversial. In histological studies, no signs of apoptotic cardiomyocytes were found in myocardium after infarction *in vivo* or *in vitro* [9]. In embryonic chick cardiomyocytes, angiotensin II does not induce apoptosis but rather prevents apoptosis [10]. ACE inhibition and angiotensin receptor blockade confers cardioprotection and improves survival in patients with heart failure [11–13]. It is possible, however, that part of the cardioprotection in heart failure conferred by blocking the renin–angiotensin system occurs through prevention of apoptotic cardiomyocyte loss.

The vasoactive peptide angiotensin II acts on its target tissues via membrane-bound receptors of the G-protein coupled family. These receptors have been divided into two subclasses: $AT_1$ and $AT_2$ [14,15]. Recently, it has been shown that stimulation of the $AT_2$ receptor antagonizes the growth effect induced by activation of the $AT_1$ receptor in cardiac cells [14]. However, there are contradictory data on the subtype of angiotensin II receptor which mediates apoptosis in myocyte and non-myocyte cells [16–18].

Therefore, we investigated whether blocking either $AT_1$ or $AT_2$ receptors can protect against apoptotic death induced by angiotensin II in neonatal rat cardiomyocyte cultures. We also studied the mechanism of the angiotensin II-induced apoptosis by measuring intracellular $Ca^{2+}$ concentrations, and by blocking L-type calcium channel activity with verapamil.

## Methods

### Cardiac cell cultures

Sprague–Dawley rat hearts (1–2 days old) were removed under sterile conditions and washed three times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced to small fragments and then agitated gently in a solution of proteolytic enzymes, RDB (Biological Institute, Ness-Ziona, Israel), which was prepared from a fig tree extract. The RDB was diluted 1 : 100 in $Ca^{2+}$ and $Mg^{2+}$-free PBS at 25°C for a few cycles of 10 min each, as described previously [19]. The mixture was centrifuged at 300 *g* for 5 min. The supernatant phase was discarded, and the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 2% chick embryo extract (Biological Industries). The suspension of the cells was diluted to $1.0 \times 10^6$ cells/ml, and 1.5 ml were placed in 35 mm plastic culture dishes on collagen/gelatin-coated cover glasses. The cultures were incubated

in a humidified atmosphere of 5% $CO_2$, 95% air at 37°C. Confluent monolayers exhibiting spontaneous contractions were developed in culture within 2 days.

### Experiments with angiotensin II and angiotensin II antagonists and verapamil

Before treatment with angiotensin II, the growth medium was replaced with chemically defined medium based on DMEM supplemented with transferrin (10 μg/ml), insulin (10 μg/ml), $T_4$ (0.1 μmol/l) and bovine serum albumin (100 μg/ml). Pretreatment with irbesartan (a selective $AT_1$ receptor antagonist), PD123319 (a selective $AT_2$receptor antagonist) and verapamil (an L-type calcium channel blocker), was accomplished by adding these compounds 20 min before exposure of cells to angiotensin II.

### α-Sarcomeric actin assay

In order to identify cardiomyocytes, cells on coverslips were stained for immunohistochemical demonstration of α-sarcomeric actin using mouse monoclonal anti α-sarcomeric actin (C-5) and goat anti-mouse biotinylated immunoglobulin conjugated with extravidin peroxidase. The chromogen 3-amino-9-ethylcarbazole (AEC) was used as described previously [19].

### Feulgen procedure

Cells on coverslips were fixed in essential fatty acid (by volume, 75 : 20 : 5 of 96% ethanol : 40% neutral formol : acetic acid) for 20 min. Fixed samples were placed in 5 N HCl for 60 min at 24°C to hydrolyse DNA and stained with the Schiff reagent as described previously [19].

### In situ apoptosis assay

Apoptotic cells were identified *in situ* using the terminal deoxynucleotidyl transferase nick-end labelling (TUNEL-like) assay as described previously [19]. After fixation in 10% neutral-buffered formalin for 20 min at room temperature and a permeabilization in 70% ethanol for 30 min at −20°C, the assay was performed on cells on coverslips using a commercial TdT FragEL DNA fragmentation KIT. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. Chromogenes AEC or DAB-Black (Zymed substrate KIT; Zymed, San Francisco, California, USA) reacted with the labelled sample to generate an insoluble colored substrate at the site of DNA fragmentation.

### Image analysis of Fuelgen and TUNEL-stained cells

The image analysis was performed with Scan-Array 2 Image Analyser (Galai, Israel). The analyser consisted of an Axiovert 135TV florescent microscope (Zeiss, Hallbergmoos, Germany) and a black and white Sony video camera, interfaced to an image analysis computer. Morphonuclear parameters were computed as described in detail previously [19]. In the present work, the system described the following parameters: AREA, morphometric parameter, which corresponds to the area of the nuclear profile; IOD, the integrated optical density, a densitometric parameter, related to the total DNA content and apoptotic index, percentage of apoptotic nuclei

### Fluorescence DNA stains

Cells were analysed for apoptosis following visualization of the fluorescent DNA-binding dye Hoechst 33342 trihydrochloride trihydrate. The monolayers were rinsed with PBS and then incubated with 10 μg/ml H33342 for 30 min. Nuclei were visualized using fluorescent microscopy and analysed for apoptotic morphology. An average of 1000 nuclei from random fields was analysed for each slide. The apoptotic index was calculated as described by Wu *et al.* [20]. At least three samples were scored per group.

### Intracellular calcium measurements

Intracellular free calcium concentration was estimated from indo-1 fluorescence, using the ratio method described by Grynkiewicz *et al.* [21]. The cardiac cells grown on coverslips were transferred to a chamber on the stage of Zeiss inverted microscope filtered with ultraviolet epifluorescence illumination. Indo-1 was excited at 355 nm and the emitted light then split by a dichroic mirror to two photomultipliers (Hamamatsu, Japan) with input filters at 405 and 495 nm. The fluorescence ratio of 405/495 nm, which is proportional to $Ca^{2+}$ concentration, was monitored. Assessment of changes in intracellular $Ca^{2+}$ concentration was performed in all experiments. In addition, intracellular $Ca^{2+}$ concentration was measured after exposure of cardiac cells to angiotensin II in a $Ca^{2+}$-free medium.

### Chemicals

Irbesartan was supplied by Bristol Myers Squibb (Princeton New Jersey, USA). Other reagents were purchased from Sigma Chemicals (St Louis, Missouri, USA).

### Statistical analysis

Results are expressed as means ± SE. ANOVA and Student's *t* test were used in statistical evaluation of the data. $P < 0.05$ was considered statistically significant.

## Results

### Characterization of neonatal cardiomyocytes in cell cultures after exposure to angiotensin II

Primary cultures of neonatal rat myocytes maintained in serum-free medium are depicted in Figure 1A. Myofibrils within these cells were elongated and uniformly distributed throughout the cell. Individual actin fibrils stained with α-sarcomeric actin antibody demonstrated the typical striated pattern. Each culture dish also contained a small number of dead cells, defined by a condensed granular cytoplasm and pyknotic nucleus. The cardiomyocytes in the angiotensin II (10 nmol/l)-treated cultures showed an increase in myofibril content and moderate hypertrophy. In preliminary experiments, myocytes were exposed to angiotensin II at concentrations of 1–1000 nmol/l for a period of 24 h (in serum-free medium) and only a weak dose-dependent response, was observed on apoptotic activity. Therefore, concentrations of 10–100 nmol/l of angiotensin II were employed in the present study. Morphological features of apoptosis in angiotensin II-treated myocytes include nuclear condensation and fragmentation, blebbing of plasma membrane, shrinking, retraction and condensation of cytoplasm (Fig. $1B_{1–4}$,C,E). Some apoptotic cells showed

also signs of mitosis and postmitotic apoptosis (Fig. 1$B_{1–4}$,E). These features were not observed in control cultures.

In control Feulgen-stained cells, chromatin was characterized by a few small granules against a pale background or abundant granules and fibrils of deeply stained chromatin (Fig. 2A). Exposure of myocytes to angiotensin II (10 and 100 nmol/l) for 24 h led to an increase in the number of apoptotic-like nuclei (Fig. 2B,C).

Histograms of the distribution of integrated optical density (IOD) versus cell number are shown in Fig. 3a–d. Nuclei stained by the Feulgen technique show four distinct populations: < 2C (hypodiploid), 2C (diploid), 4C (tetraploid) and ⩾ 8C. For each of these populations, there is a constant value for IOD, despite a wide variation in nuclear size. In control cultures individual interphase nuclei, the $G_0/G_1$ population, which covered the 1.8–2.4C range of DNA content were predominant. A second peak was recorded in the range of 2.5–4.9C corresponding to cells in S-phase and tetraploid cells ($G_2$/M). The amount of nuclei with hypodiploid DNA content (below the range of 2C) in control cultures was no more than 2–3% (Fig. 3a). The histogram of DNA content in angiotensin II-treated cells demonstrated a 4.1-fold increase in hypodiploid peak over control cells and no differences in ⩾ 2C DNA distribution (Fig. 3b,c). It is evident that the differences between angiotensin II-treated and control cells are due to the smaller nuclear area and low DNA content of hypodiploid, Feulgen-stained cells, in spite of the variability in nuclear area.

The apoptotic index (percentage of apoptotic nuclei) of Feulgen-stained cells is shown in Figure 4. Angiotensin II (10–100 nmol/l) caused a three-fold increase in apoptotic cells (from 2.4 ± 1.3% in the control cells to 6.3 ± 3.3% and 7.6 ± 2.8% in the 10 and 100 nmol/l of angiotensin II-treated cells, respectively, $P < 0.05$).

Analysis of the fluorescent morphology of Hoechst-stained cells shows that angiotensin II (10–100 nmol/l) caused a marked increase in apoptotic cell death (from 2.6 ± 0.65% in the control cells to 8.8 ± 2.9% and 10.2 ± 2.6% in the 10 and 100 nmol/l of angiotensin I-treated cells, respectively, $P < 0.05$) (Fig. 4).

To further examine the cells that contained fragmented nuclear DNA typical of apoptosis, we performed an *in situ* assay based on end-labelling of DNA strand breaks by the TUNEL-like method (Fig. 5). TUNEL positive cells were obtained following Ang II treatment (Fig. 5B) confirming our results with Fuelgen and Hoechst stainings. These TUNEL positive cells were shown to be cardiomyocytes (and not fibroblasts) as demonstrated by the double staining with both TUNEL and α-sarcomeric actin (Fig. 5C). The apoptotic index of TUNEL-stained cells is shown in Figure 4. Angiotensin II (10–100 nmol/l) caused a four-fold increase in apoptotic cell death (from 1.4 ± 0.28% in the control cells to 5.8 ± 1.24% and 6.5 ± 1.54% in the 10 and 100 nmol/l of angiotensin II-treated cells, respectively, $P < 0.05$).

### Effects of angiotensin II antagonists on cardiomyocyte death

Exposure of cardiac cells to the $AT_1$ selective antagonist irbesartan (100 nmol/l) or to the $AT_2$ receptor antagonist PD123319 (1 μmol/l), alone had no effect on apoptosis (data not

shown). Treatment of cardiac cells with irbesartan (100 nmol/l) completely prevented angiotensin II-mediated apoptosis (Figs 1D, 3d and 5D). Similar results were obtained with PD123319 (1 μmol/l) (Fig. 2D), indicating the presence of both receptors on the cardiomyocytes. Apoptotic indices derived from Feulgen, Hoechst and TUNEL-like assays are shown in Figure 4.

### Effects of the calcium channel blocker verapamil on cardiomyocyte death

Exposure of cardiac cells to verapamil (10 μmol/l) alone had no effect on apoptosis (data not shown). Pretreatment of cardiac cells with verapamil completely prevented angiotensin II-mediated apoptosis (Fig. 1F).

### Effects of angiotensin II and angiotensin II antagonists on intracellular $Ca^{2+}$ concentrations

Exposure of cardiomyocytes to angiotensin II (1–200 nmol/l) caused an increase in the concentration of intracellular free $Ca^{2+}$ in a concentration-dependent manner. The drug produced a rapid rise followed by a sustained increase in $Ca^{2+}$ that lasted 40–60 s (Fig. 6a). Treatment of cells in a $Ca^{2+}$-free medium did not abolish the intracellular $Ca^{2+}$ rise induced by angiotensin II, indicating that $Ca^{2+}$ elevation following response to angiotensin II arises from intracellular stores (not shown). Pretreatment of cells with the $AT_1$ antagonist irbesartan (100 nmol/l) completely blocked the angiotensin II-induced increase in intracellular calcium (Fig. 6b). Pretreatment of cardiomyocytes with the $AT_2$ antagonist PD123319 at a concentration of 1 μmol/l only mildly attenuated the intracellular $Ca^{2+}$ rise but 10 μmol/l of the antagonist completely blocked elevation in intracellular free calcium induced by angiotensin II (Fig. 6c).

## Discussion

In the present study, we demonstrated that angiotensin II induces apoptosis in neonatal rat cardiomyocytes which had been grown in culture for 5–6 days. Recently, using the terminal deoxytransferase dUTP nick-end labelling (TUNEL-like) analysis, it has been shown that angiotensin II induces apoptosis in cardiac myocytes [6–8,18] and many other cells [16,17,22,23]. The scoring of apoptotic cells based on the TUNEL-like assays was found to be very ambiguous because it does not discriminate between apoptotic, necrotic and autolytic cells [9,24]. Our results were confirmed by various methods of analysis, including careful morphological and immunocytochemical investigation of dying cardiomyocytes.

At present, the function of the $AT_2$ receptors in cardiomyocytes is still not well defined. Stimulation of $AT_2$ receptors has been demonstrated to inhibit $AT_1$-receptor-dependent growth [25,26], and to induce apoptosis in cultured neonatal rat cardiomyocytes [27]

To establish whether angiotensin II-induced myocytes apoptosis is mediated by activation of the $AT_1$ or $AT_2$ receptor subtypes, we used irbesartan and PD123319, as receptor antagonists. Pretreatment with the selective $AT_1$ antagonist irbesartan, before exposure, conferred a full protection against the apoptotic effect of angiotensin II, indicating that angiotensin II induces apoptosis through activation of the $AT_1$ receptor. Similar results have

been previously described with another non-peptide $AT_1$ antagonist, losartan [6,7], but this is the first documentation that irbesartan can also prevent angiotensin II-induced apoptosis.

Recently, it has been shown, in rat new-born cardiomyocytes, that angiotensin II-induced apoptosis was prevented by pretreatment with $AT_1$ antagonist, but not by the $AT_2$ antagonist PD123319 [6]. Similar findings were demonstrated by Kajstura *et al.* in adult (3 months) rat cardiomyocytes [7]. Nevertheless, in-vitro experiments suggested that $AT_2$ type receptors were involved in apoptosis of non-myocyte cells [23,28]. Horiuchi *et al.* have shown that activation of $AT_2$ receptors in cardiomyocytes inhibits cellular growth and exerts a proapoptotic effect by antagonizing the effects of the $AT_1$ receptors [16]. Moreover, it has been demonstrated recently that adult spontaneous hypertensive rats (SHR) exhibit increased susceptibility to angiotensin II-induced apoptosis and that blockade of both the $AT_1$ and the $AT_2$ receptor blunted the apoptotic response to angiotensin II [29]. We have found that activation of both $AT_1$ and $AT_2$ receptors is necessary for angiotensin II to mediate apoptosis. Both receptor subtypes have a role in cardiovascular development and remodelling. The $AT_1$ receptor stimulates cell proliferation, which is associated with sustained mitogen-activated protein (MAP)-kinase activation. The $AT_2$ receptor inhibits growth and induces apoptosis in cultured cells and *in vivo* through activation of tyrosine phosphotase(s), such as MAP-kinase-phosphatase, resulting in Bcl-2 dephosphorylation (an inhibitor of apoptosis) and upregulation of Bax (an inducer of apoptosis). The mitogenic response induced by angiotensin II is associated with sustained MAP-kinase activation [28,30]. Despite the strong evidence that the $AT_1$ and $AT_2$ receptors play different roles in the regulation of apoptosis in other cell types, in which $AT_1$ receptor activation inhibits apoptosis and $AT_2$ receptor activation exerts antigrowth and proapoptotic effects, it has been shown that in cardiomyocytes the $AT_1$ receptors behave differently and are involved in the promotion of apoptosis [6,7,31].

The role of angiotensin II in inducing apoptosis was also demonstrated *in vivo*. In SHR, chronic blockade of $AT_1$ receptors was found to prevent Bax oncoprotein overexpression and to normalize apoptosis in the left ventricle. The effect was shown to be independent of the haemodynamic effects of $AT_1$ blockade [8]. In a model of dogs with moderate heart failure (ejection fraction 30–40%), it was also demonstrated that early long-term monotherapy with an ACE inhibitor significantly attenuates cardiomyocyte apoptosis [32].

The apoptotic effects of angiotensin II in a normal healthy heart are unknown. However, in pathological conditions, such as heart failure, a reduction in the extent of cardiomyocyte apoptosis may be one mechanism by which ACE inhibitors and $AT_1$ antagonists preserve global left ventricular function. It has also been suggested that apoptosis might be a mechanism involved in the reduction of cardiomyocyte mass that accompanies the transition from stable compensation to heart failure in hypertensive heart disease [33].

The effect of angiotensin II on intracellular $Ca^{2+}$ concentration was immediate and lasted 40–60 s. Because treatment of cells in a calcium-free medium failed to prevent the intracellular $Ca^{2+}$ elevation induced by angiotensin II, it can be concluded that $Ca^{2+}$ arise from intracellular stores (e.g. the sarcoplasmic reticulum). The calcium rise induced by angiotensin II was concentration-dependent and had an effect on the whole population of

cultured myocytes. Shao *et al.* [34], demonstrated that exposure of cardiomyocytes to angiotensin II (0.01–10 μmol/l) resulted in an immediate and sustained increase in intracellular $Ca^{2+}$ in a concentration-dependent manner. This increase was blocked by antagonists of both $AT_1$ and $AT_2$ receptors. In contrast, in the experiments performed by Touyz *et al.*, where only a low PD123319 concentration was employed, the $AT_2$ antagonist had no effect on $Ca^{2+}$ transience [35]. Our results are in accordance and explain both observations.

The calcium rise induced by angiotensin II was concentration-dependent, while angiotensin II-induced apoptosis was not concentration-dependent. Pretreatment with the $AT_1$ antagonist irbesartan prevented both apoptosis and $Ca^{2+}$ rise, whereas, pretreatment with the $AT_2$ antagonist PD123319 at a concentration of 1 μmol/l prevented angiotensin II-induced apoptosis but had little effect on the angiotensin II-induced rise in intracellular $Ca^{2+}$. Thus, it seems that angiotensin II-induced apoptosis is not related to the immediate intracellular $Ca^{2+}$ rise induced by angiotensin II.

We have demonstrated that the apoptotic process induced by angiotensin II was associated with cardiomyocyte replication, was not dose-dependent and occurred only in a small group of cardiomyocytes. Therefore, we can assume that the number of receptors is not the limiting factor for angiotensin II-induced apoptosis. Only cells that undergo mitosis are susceptible to apoptosis. This observation is supported by our finding that verapamil completely prevented cardiomyocyte proliferation and angiotensin II-induced apoptosis. Calcium channel blockers have been shown to inhibit cell replication [36,37]; thus, both cardiomyocyte proliferation and post-mitotic apoptosis are prevented with the use of verapamil.

Our morphological results can explain why blockade of independent $AT_1$ and $AT_2$ receptor subtypes inhibits the proapoptotic effects of angiotensin II. Activation of the $AT_1$ receptor is needed for cell replication and $AT_2$ receptor activation induces mitotic arrest and apoptotic expressions only on replicated cells. Therefore, stimulation of both receptor types is required for the apoptotic effects of angiotensin II, and blockade of either $AT_1$ or $AT_2$ receptor subtypes inhibits the proapoptotic effects of angiotensin II.

Recognition of the factors responsible for the initiation or prevention of programmed cell death may eventually lead to therapeutic interventions.

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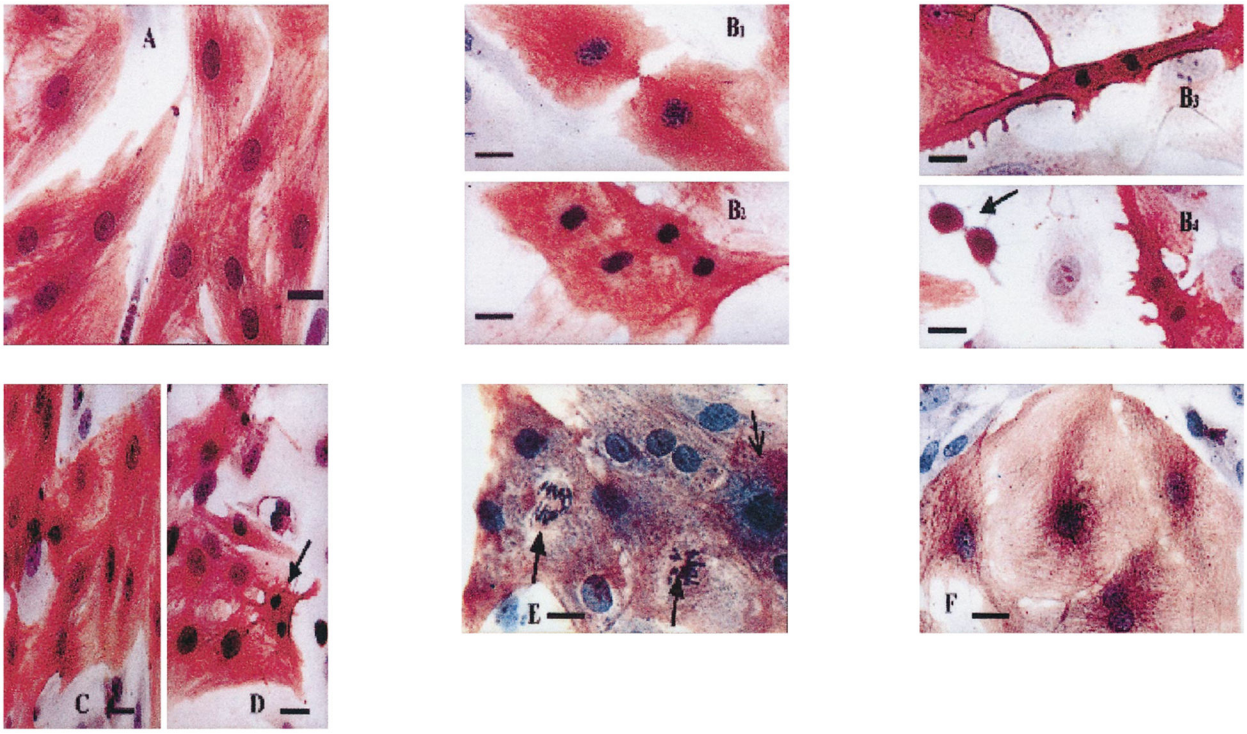


**Fig. 1.**

Morphology of cardiocytes following angiotensin II treatment. Cardiac cells 3 days in culture were transferred to serum-free media for 2 days before treatment with angiotensin II (10 nmol/l) for 24 h. The cells were stained with α-saromeric actin and counter-stained with haematoxylin. (A) Control cells. (B) Angiotensin II (10 nmol/l)-treated cells. Some myocardial cells entered the mitotic stage and the late telophase reappearance of myofibrils, decondesation of chromosomes and appearance of double nuclear membrane were observed ($B_1$). Many myocytes in late telophase were characterized by further condensation of chromosomes to uniformly and tightly packed chromatin figures, shrinking, retraction, condensation of cytoplasm, loss of organized myofibrils and reappearance of nuclear membrane ($B_{2–3}$). After the end of cleavage, most myocytes became typical apoptotic bodies ($B_4$). (C) Angiotensin II (100 nmol/l)-treated cells. (D) Pretreatment with irbesartan (100 nmol/l) before exposure to angiotensin II (10 nmol/l). Arrows indicate apoptotic cardiomyocytes. (E,F) Experiments with verapamil. The cells were stained with α-saromeric actin and counter-stained with haematoxylin. (E) Angiotensin II (10 nmol/l)-treated cells. Cardiomyocytes undergoing mitosis (wide arrows) and apoptosis (narrow arrow) are demonstrated. (F) Pretreatment with verapamil (10 μmol/l) before exposure to angiotensin II (10 nmol/l). Bars = 10 μm.

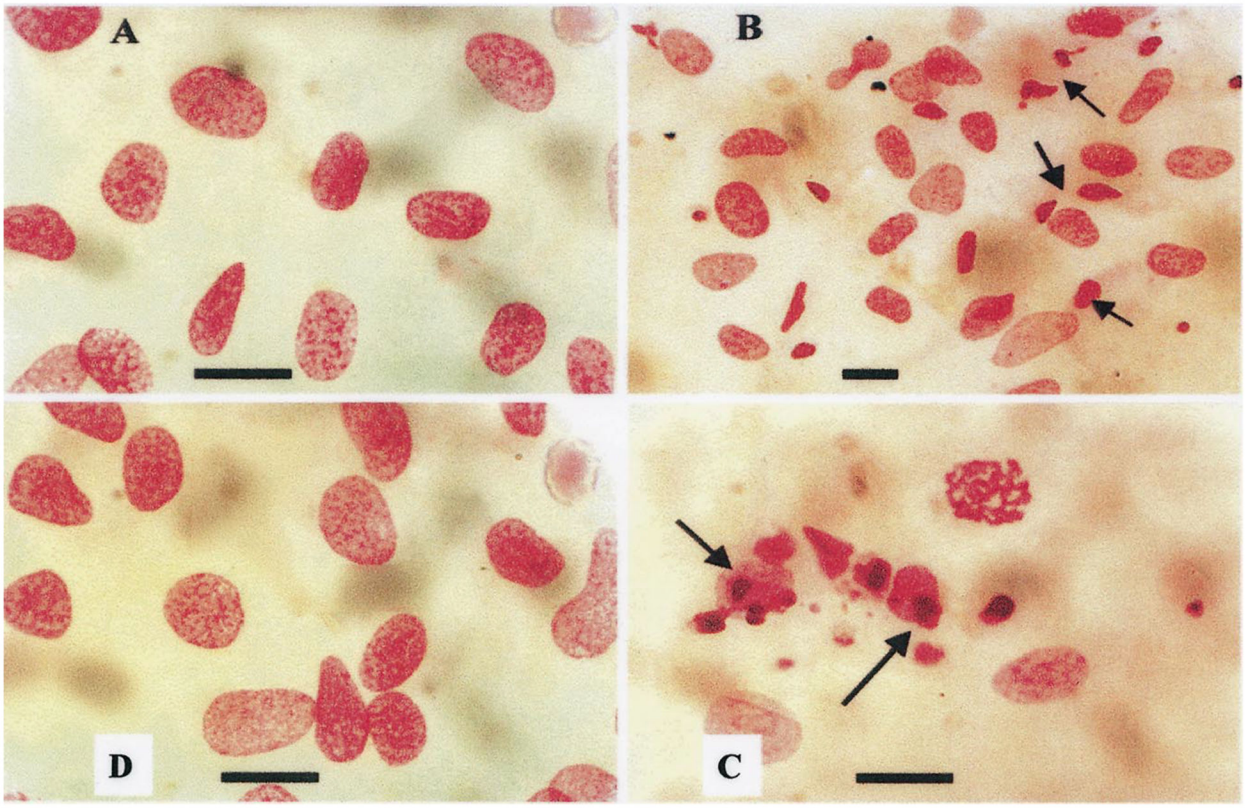


**Fig. 2.**

Morphology of nuclei of Feulgen-stained cultured cardiomyocytes after exposure to angiotensin II. (A) Nuclei of control cells. A few small granules against a pale background characterizes chromatin. (B) Exposure of myocytes to angiotensin II (10 nmol/l) for 24 h led to an increase in the number of apoptotic nuclei. Condensation, compacting and margination of nuclear chromatin were accompanied by disappearance of the structural framework of the nucleus and nuclear breakdown. (C) Exposure of myocytes to angiotensin II (100 nmol/l). (D) Pretreatment with PD123319 (1 μmol/l) before exposure to angiotensin II (10 nmol/l). Arrows indicate apoptotic cardiomyocytes. Bars = 10 μm.

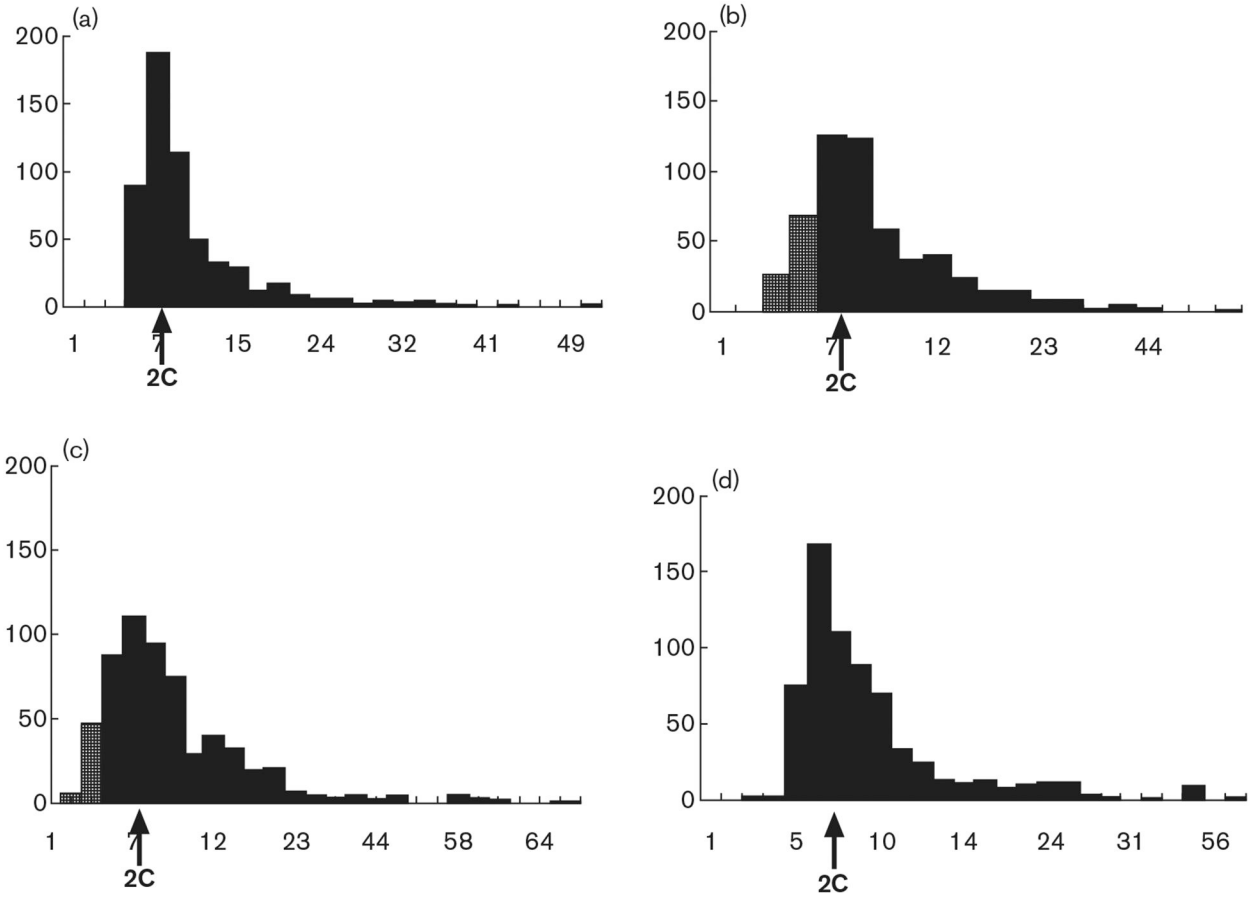


**Fig. 3.**

Image analysis of Feulgen-stained nuclei following exposure to angiotensin II. Histograms of DNA content are demonstrated (arbitrary units of integrated optical density, IOD). (a) Control. (b) 24 h after treatment with angiotensin II (10 nmol/l). (c) 24 h after treatment with angiotensin II (100 nmol/l). (d) Pretreatment with irbesartan (100 nmol/l) before angiotensin II (10 nmol/l) (strippled bars indicate hypoploid DNA content). 2C (arrow), normal, diploid content of DNA.

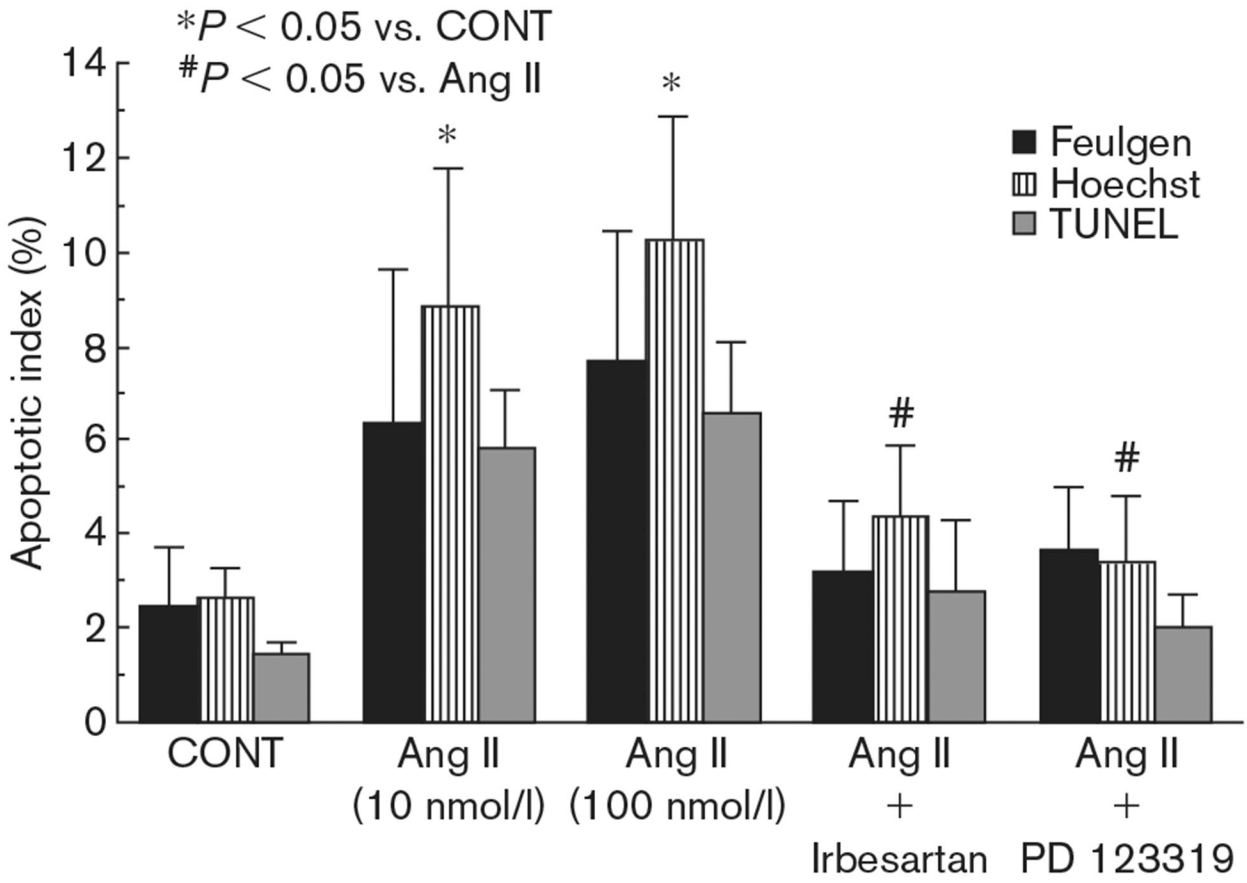


**Fig. 4.**

Apoptotic index of Feulgen, Hoechst and TUNEL- stained myocytes after angiotensin II treatment. Cardiocytes were treated with the indicated drugs 20 min before application of angiotensin II (10 nmol/l). Feulgen, Hoechst and TUNEL staining were performed 24 h later. CONT, control; Ang II, angiotensin II. Irbesartan, 100 nmol/l; PD123319, 1 μmol/l. Angiotensin II was employed at a concentration of 10 nmol/l except when indicated.

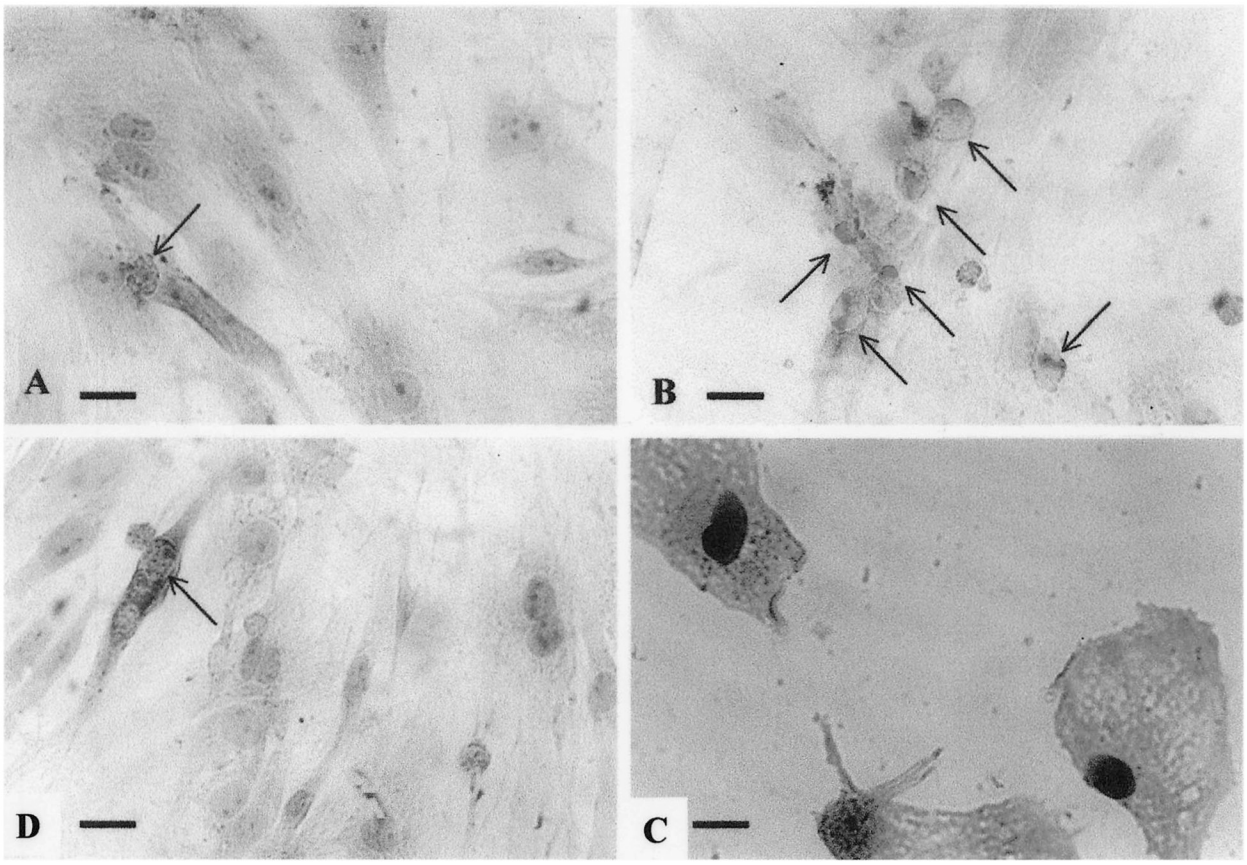


**Fig. 5.**

Immunohistochemical demonstration of DNA breaks (TUNEL-like) following angiotensin II treatment. Cardiac cells 3 days in cultures were transferred to serum-free media for 2 days before treatment with angiotensin II (10 nmol/l) for 24 h. (A) Control. (B) Angiotensin II (10 nmol/l)-treated cells. (C) Angiotensin II (10 nmol/l)-treated cells. Double staining: 3′-hydroxyl-free ends of DNA (DAB-black) and α-sarcomeric actin (AEC-red). (D) Pretreatment with irbesartan (100 nmol/l) before angiotensin II (10 nmol/l). Arrows indicate apoptotic cardiocytes. Bars = 1 μm.

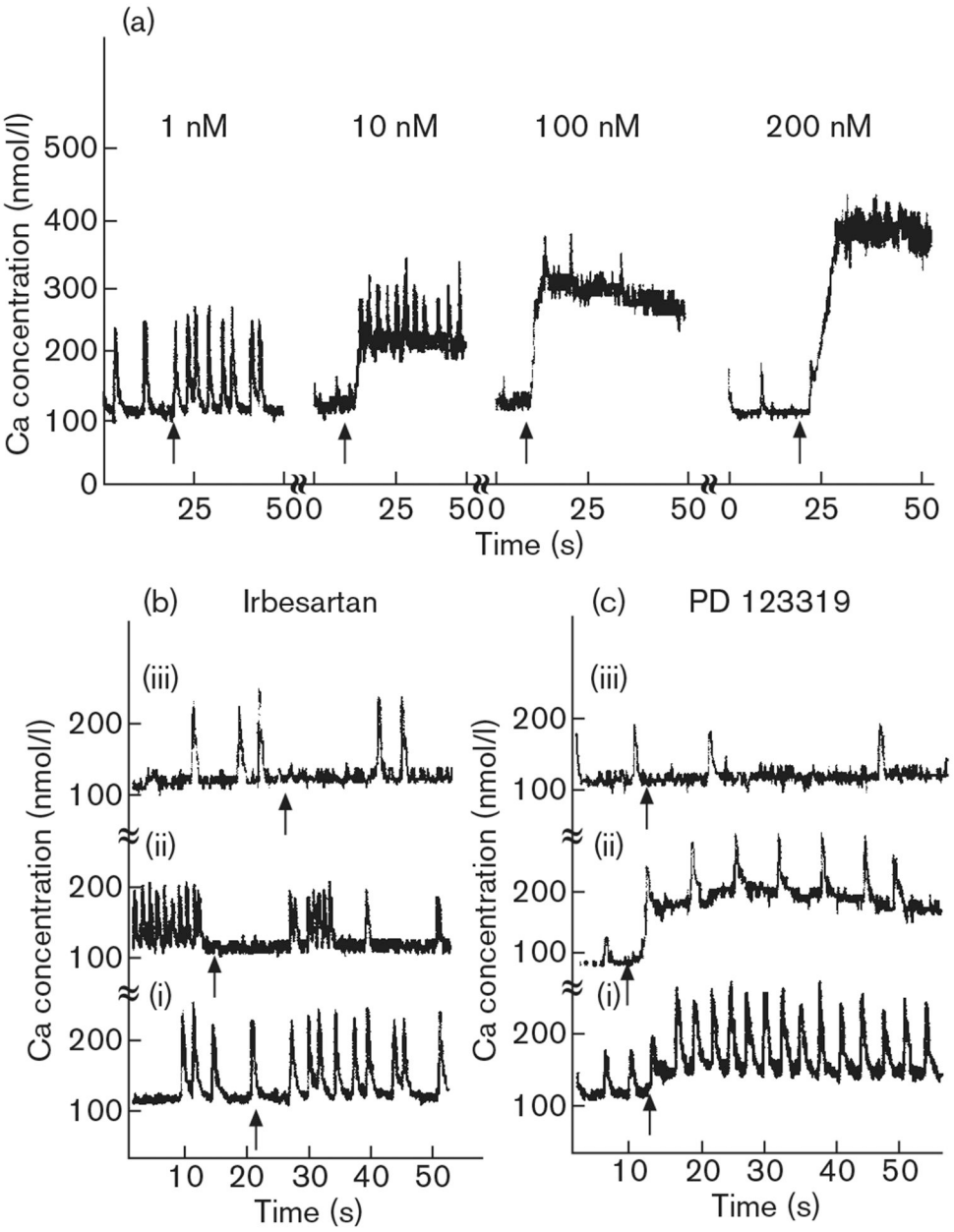


**Fig. 6.**

Effect of angiotensin II on intracellular free calcium in cultured rat cardiomyocytes. (a) Effects of various concentrations of angiotensin II on intracellular calcium transience. Typical tracings are shown. (b) Effect of the selective angiotensin II $AT_1$ receptor antagonist irbesartan on the change in intracellular free calcium induced by angiotensin II (arrows): (i) irbesartan, 100 nmol/l, angiotensin II, 10 nmol/l; (ii) irbesartan, 100 nmol/l, angiotensin II, 100 nmol/l; (iii) irbesartan 1 μmol/l, angiotensin II, 100 nmol/l. (c) Effect of the selective angiotensin II $AT_2$ receptor antagonist PD123319 on the change in intracellular free calcium induced by angiotensin II (arrows): (i) PD123319, 1 μmol/l, angiotensin II, 10 nmol/l; (ii) PD123319, 1 μmol/l, angiotensin II, 100 nmol/l; (iii) PD123319, 10 μmol/l, angiotensin II, 100 nmol/l. Cultures were incubated with antagonists for 5 min at room temperature prior to

the addition of angiotensin II. Similar results were observed in five independent experiments.