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Angiotensin II via AT_1 receptors may mediate apoptosis in the cardiac conduction system of rats

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Apoptosis has been suggested as a possible cause of gradual development of complete heart block and fatal arrhythmias associated with absence of the AV node, sinus, and internodal pathways (James *et al,* 1996). Studies about apoptosis in the heart by means of cardiomyocyte cell culture have demonstrated that angiotensin II (Ang II) mediates cardiomyocyte apoptosis via angiotensin II type I receptors (AT_1) (Cigola *et al*, 1997). The transgenic *m(Ren-2)27* (TG) rat carries the additional *Ren-2* gene, the expression of which results in an increase of heart Ang II (Campbell *et al,* 1995), thus potentially affecting the cell growth/death equilibrium. This study addresses the question of role of Ang II/AT_1 receptors mediated apoptosis in the sinoatrial (SA) and atrioventricular nodes (AV).

Six, male 2 week TG and Hannover Sprague Dawley (SD) rats were anaesthetised by pentobarbitone sodium i.p. injection (100 mg/kg). The hearts were removed and fixed in 10% formaldehyde. Following dehydration and embedding in paraffin, 5 μm serial sections were cut then stained with Masson Trichrome to localize SA and AV nodes. The sections containing SA or AV node were processed for either: (a) calculation of apoptotic nuclei following terminal deoxnucleotidyl transferase nick end labelling of 3'-OH ends using Fluorescein-FragEL™; or (b) immunohistochemical labelling with antibodies to the AT_1 receptors prior to confocal scanning laser microscopical analysis. Quantification of AT_1 receptors was performed by using Microimage analysis software (Olympus).

Group	Apoptotic cells/mm^2		AT_1 receptors ($\times 10^3$)/mm^2	
	SA	AV	SA	AV
SD	0.040±0.07	0.164±0.12	1.14±0.17	7.63±1.91
TG	**0.140±0.37***	**0.433±0.11***	**1.67±0.26***	**12.50±3.97***

Data expressed as mean ± SD (n=6)
* = significant compared with control (P<0.05) (Independent-Sample T-test)

The table shows that the number of apoptotic cell in both the SA and AV node is significantly greater in the TG compared with the SD (p<0.05). Quantification of AT_1 receptors within SA and AV node shows that there were significantly more AT_1 receptors in the TG compared with the SD (p<0.05). These data suggest that an elevated level of apoptosis in the TG rat heart compared with the controls could be accounted for by *Ren-2* derived Ang II active via AT_1 receptors.

Campbell, D.J., Rong, P., Kladis, A., Rees, B., Ganten, D. and Skinner, S.L.(1995) Angiotensin and Bradykinin peptides in the TGR (mRen-2)27 rat. *Hypertension*, 25, 1014-1020.
Cigola, E., Kajstura, L.B., Meggs, L.G. and Anversa, P. (1997) Angiotensin II activates programmed myocyte cell death *in vitro*. *Experimental Cell Research*, 231, 363-371.
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Angiotensin II may mediate apoptosis via the AT_1 receptors in the rat cardiac conduction system

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Abstract

Introduction: Apoptosis and angiotensin II (Ang II) have been suggested as possible causes of arrhythmias. In addition, Ang II via angiotensin II type I receptors (AT_1) has been demonstrated to induce cardiomyocyte apoptosis. The transgenic m(Ren-2)27 (TG) rat carries the additional Ren-2 gene, the expression of which results in an increase of heart Ang II thus potentially affecting the cell growth/death equilibrium. In this research we have studied the effect of angiotensin II (Ang II) via the AT_1 receptors on mediating apoptosis in a cardiac conduction system (SA node and AV nodes). **Materials and methods**: Heart sections from male 2-day, 1-week and 2-week TG and Sprague Dawley (SD) rats were stained with Masson Trichrome to localize SA and AV nodes. The sections containing SA or AV nodes were processed for quantitation of apoptotic nuclei and AT_1 receptors. **Results**: The number of apoptotic nuclei/mm^2 in the SA and AV nodes were found to decrease from 2-day to 2-week in both the TG and the SD rats, and the number of apoptotic nuclei/mm^2 in the TG groups was significantly higher than that of the SD groups for all ages ($p<0.05$). The number of AT_1 receptors/mm^2 in the SA node were found to decrease with increasing age, whereas the number of AT_1 receptors/mm^2 in the AV node was increased in both TG and SD rats and the number of AT_1 receptors/mm^2 in the three TG groups was significantly more than that of the three SD groups ($p<0.05$). **Discussion and Conclusion**: As a consequence of the additional renin gene in the TG rats , which results in the alteration of the local renin-angiotensin system, the numbers of AT_1 receptors/mm^2 and apoptotic nuclei/mm^2 are increased. The number of apoptotic nuclei/mm^2 decrease with maturation, therefore, the apoptosis may not be used to explain the cause of the arrhythmia in the aging patient. However, the number of AT_1 receptors in the AV node, of which there are more than in the SA node at 2-week rats, could be used to explain the pathology of arrhythmia associated with angiotensin in hypertension patients.

Key words : Angiotensin II, apoptosis, conduction system, AT_1 receptor.

Introduction

Cardiac arrhythmias are a major health problem in cardiomyopathies. They cause severe symptoms and even sudden death (Myerburg et al, 1993) In addition, there is no treatment available to prevent sudden death in patients with arrhythmia (De Mello, 2001). Cardiac arrhythmias are caused by multiple mechanisms such as abnormal automaticity after depolarization and reentry (De Mello, 2001). It has been suggested that angiotensin II (Ang II) possesses arrhythmogenic activity such as decrease in cell coupling and reduction in conduction velocity, facilitating the generation of reentrant rhythms. (De Mello and Altien, 1992; De Mello, 1996:, De Mello and Danser, 2000, ; De Mello, 1998 and De Mello, 1994). In addition, apoptosis has been suggested as a cause of gradual development of complete heart block and fatal arrhythmia, associated with absence of the AV node, sinus and internodal pathways (Jame et al, 1996). Studies about apoptosis in the heart by means of cardiomyocyte cell culture (in vitro) have demonstrated that Ang II mediates cardiomyocyte apoptosis via angiotensin II type I receptors (AT_1) (Cigola et al, 1997; Kajstura et al, 1997). However, in the in vivo condition, it is not known whether Ang II is involved in inducing apoptosis in the heart, and in particular in the conduction system.

The m(Ren-2)27 transgenic rats (TG) are rats carrying the additional mouse Ren-2 renin gene. The rats develop fulminant hypertension despite low circulating renin. Therefore, it is hypothesized that hypertension results from enhanced local Ang II generation in extrarenal tissue (Lee et al, 1995). The renin gene is strongly expressed in extrarenal tissue especially in the heart, where the increase of heart Ang II is found (Campbell et al, 1995). Due to the increased Ang II in the heart, the cell growth/death equilibrium may potentially be affected. This study focusses on the role of Ang II via AT_1 receptors in mediating apoptosis in the cardiac conduction system (sinoartial node, SA, and atrioventricular node, AV) in the in situ condition by comparing the apoptosis level and number of AT_1 receptors between transgenic rats and normal Sprague Dawley rats. The study is performed in postnatal rats (2 days, 1 week and 2 weeks) as these stages have been suggested to be important for morphogenesis of the conduction system (Jame, 1994). Results from this study may explain the pathology of arrhythmia and may lead to new approaches to prevent disaster or to improve arrhythmia therapies.

Materials and Methods

1. Animals

The m(Ren-2)27 transgenic rat (TG) and Sprague Dawley (SD) rats were obtained from the Biomedical Service Units, School of Biomedical Sciences, Nottingham University, UK (provided by Dr T. Bennett and Dr S.M Gardiner). The rats were kept on a standard laboratory diet with free access to food and water on a 12:12 h light/dark cycle at 22°C.

All 2-day, 1-week and 2-week male TG and SD rats (six rats per group, total 36 rats) were anesthetized by intraperitoneal injection of pentobarbitone sodium (100mg/kg). The hearts were removed and fixed with 10% formalin. Following dehydration and paraffin wax embedding, 5 μm serial sections were cut and mounted on TESPA coated slides. Random sections were stained with Masson Trichrome to localize the SA and AV nodes.

2. Quantitative analysis of apoptotic nuclei in the SA and AV nodes

2.1 *In situ* end labeling procedure

Every tenth section containing the SA node or the AV node of each heart were systematically sampled (Mayhew, 1991) for in situ terminal deoxytransferase nick end labeling using the Fluorescent FragEL™ DNA Fragmentation Detection kit (Oncogene Research Products). The sections were deparaffinized and dehydrated before permeabilization with 20 μg/ml proteinase K in 10 mM Tris for 20 minutes. A positive control was generated by incubating with 1 μg/μl DNase I in 100 mM Tris, 700 mM NaCl pH 7.6 for 20 minutes. After incubating with Equilibration Buffer (1 M sodium cacodylate, 0.15 M Tris, 1.5 mg/ml BSA, 3.75 mM $CoCl_2$ pH 6.6) for 10-30 minutes, the sections were incubated with Labeling Reaction Mixture Comprising FITC–conjugated deoxynucleotide and terminal deoxynucleotide transferase (TdT), except one slide, a negative control which was incubated with the labeling mixture without TdT, at 37°C for 1 hour. The sections were washed, then mounted using Fluorescein-FragEL™ Mounting Media.

2.2 Quantitative analysis of apoptotic nuclei

Quantitative analysis of apoptotic nuclei stained with Fluorescein–FragEL™ was performed by using a fluorescent microscope with an excitation wavelength of 494 mm. Apoptotic nuclei identified by morphological criteria and intensity of staining were counted. The number of apoptotic nuclei was calculated by dividing the total number of apoptotic nuclei by the area of sections examined. Microimage analysis software (Olympus) was applied to estimate the

area of sections examined in the SA node and in the AV node. The results were expressed as number of apoptotic nuclei per mm^2.

3. Quantitative analysis of AT_1 receptors in the SA and AV nodes

3.1 Immunofluorescence

Every tenth section adjacent to the sections stained for apoptosis was systematically sampled (Mayhew, 1991). Immunofluorescence was performed to localize the number of AT_1 receptors in the SA and AV nodes. After deparaffinizing and rehydration, the sections were pre-incubated in 0.3%, Triton X-100, in 0.01 M potassium phosphate buffer saline (KPBS) for 30 minutes and a blocking solution (1.5% goat serum in KPBS) for 20 minutes prior to incubation in the primary antibody, rabbit polyclonal IgG raised against AT_1 receptors (Autogen Bioclear), at 1:50 dilution $4^{o}C$ overnight, followed by the secondary antibody, 1:200 FITC-labeled goat anti-rabbit IgG (Vector laboratories) for 2 hours. Finally the sections were mounted using Vactashield mounting medium (Vector laboratories). Negative controls were obtained by replacing the primary antibody with diluting buffer.

3.2 Quantitative analysis of AT_1 receptors

The sections were enlarged under confocal laser scanning microcopy for visualizing AT_1 receptors and photographing. The AT_1 receptors in each photograph were counted by Microimage analysis software (Olympus). The results were expressed as number of fluorescent AT_1 spots per mm^2

4. Statistical Analysis

Data are reported as mean $\pm$ SEM. Statistical analysis was performed by Student's T-test to compare the number of apoptotic nuclei/mm^2 and AT_1 receptors/mm^2 between SD and age-matched TG. Statistical significance was set at $P<0.05$.

Results

1. Localization the SA and AV nodes

Masson Trichrome staining showed the sinoatrial node (SA) at the wall of the proximal superior vena cava adjacent to the right atrium. SA nodal cells consisting of thin modified muscle cells are stained more lightly than those of cardiomyocytes (Fig 1A-B). The coronal sections of the heart stained with Masson Trichrome showed the atrioventricular node (AV) at the lower part of the interatrial septum astrides the interventricular septum. The AV node cells are smaller in diameter and stained more lightly than those of ventricular cardiomyocytes (Fig 1C-D).

2. Quantitative analysis of apoptotic nuclei in the SA and AV node

2.1 Morphology of apoptotic nuclei

The nuclei labeled for internucleosomal strand breaks using Fluorescein-FragEL™ Kit were viewed under fluorescent microscopy and appeared as small, brightly-stained nuclei exhibiting morphological features of chromatin condensation, characteristic of apoptotic nuclei (Fig 2A-D).

2.2 Quantitative analysis of apoptotic nuclei in the SA node

Quantitative analysis of Fluorescein-FragEL™ labeled nuclei showed that in all three age-groups, the number of apoptotic nuclei was significantly higher in the TG rats (2-day 1.48 ± 0.75 cell/mm^2; 1-week 0.66 ± 0.33 cell/mm^2 and 2-week 0.13 ± 0.07 cell/mm^2) compared with the SD rats (2-day 0.77 ± 0.60 cell/mm^2; 1-week 0.14 ± 0.22 cell/mm^2 and 2-week 0.04 ± 0.07 cell/mm^2) ($P = 0.002$, 0.04 and 0.035 for 2-day, 1-week and 2-week, respectively). The number of apoptotic nuclei was reduced with maturation in both the TG and SD rats (Fig 3).

2.3 Quantitative analysis of apoptotic nuclei in the AV node

Quantitative analysis of Fluorescent – FragEL™ labeled nuclei showed that in all age-groups, the number of apoptotic nuclei was significanty higher in the TG rats (2-day 5.27 ± 2.60 cell/mm^2; 1-week 2.23 ± 0.08 cell/mm^2 and 2-week 0.43 ± 0.11 cell/mm^2) than those of the SD rats (2-day 2.68 ± 2.96 cell/mm^2; 1-week 0.26 ± 0.4 cell/mm^2 and 2-week 0.16 ± 0.12 cell/mm^2) ($P = 0.01$, 0.04 and 0.032 for 2-day, 1-week and 2-week respectively). The number of apoptotic nuclei was reduced with maturation in both the TG and SD rats (Fig 4).

3. Quantitative analysis of AT_1 receptors in the SA and AV node

3.1 Immunofluorescence

Immunofluorescent labeled sections with anti–AT_1 receptors viewed under the confocal laser scanning microscope showed the distribution of the AT_1 immunoreactivity (the bright-green fluorescent dots) in the SA and AV nodes (Fig. 5A-F and 6A-F).

3.2 Quantitative analysis of AT_1 receptors in the SA node

In the SA nods, the number of AT_1 receptors at all three age-groups of the TG rats were $14.05 \pm 2.05 x 10^{-3}/mm^2$, $7.42 \pm 1.32 x 10^3/mm^2$, $1.67 \pm 0.26 x 10^3/mm^2$, respectively, which were significantly higher than those of the SD rats (2-day $7.05 \pm 1.14 x 10^3/mm^2$; 1-week $2.92 \pm 0.67 x 26 x 10^3/mm^2$ and 2-week $1.14 \pm 0.17 x 10^3/mm^2$) (P=0.032, 0.02, 0.011 for 2-day, 1-week and 2- week, respectively). The number of AT_1 receptors was down–regulated from 2-day to 2-week in both the TG and the SD rats (Fig 7).

3.3 Quantitative analysis of AT_1 receptors in the AV node

In the AV node, the numbers of AT_1 receptors at all three age-groups of the TG rats were $5.26 \pm 1.60 x 10^3/mm^2$, $12.13 \pm 2.03 x 10^3/mm^2$, $12.50 \pm 3.97 x 10^3/ mm^2$ respectively, which were significantly higher than those of the SD rats (2-day $2.61 \pm 0.49 x 10^3/mm^2$; 1-week $5.37 \pm 1.20 x 10^3/mm^2$, and 2-week $7.63 \pm 1.91 x 10^3/mm^2$) (P = 0.031, 0.04 and 0.006 for 2-day, 1-week and 2-week, respectively). The number of AT receptors was up–regulated from 2-day to 2-week in both the TG and the SD rats (Fig 8).

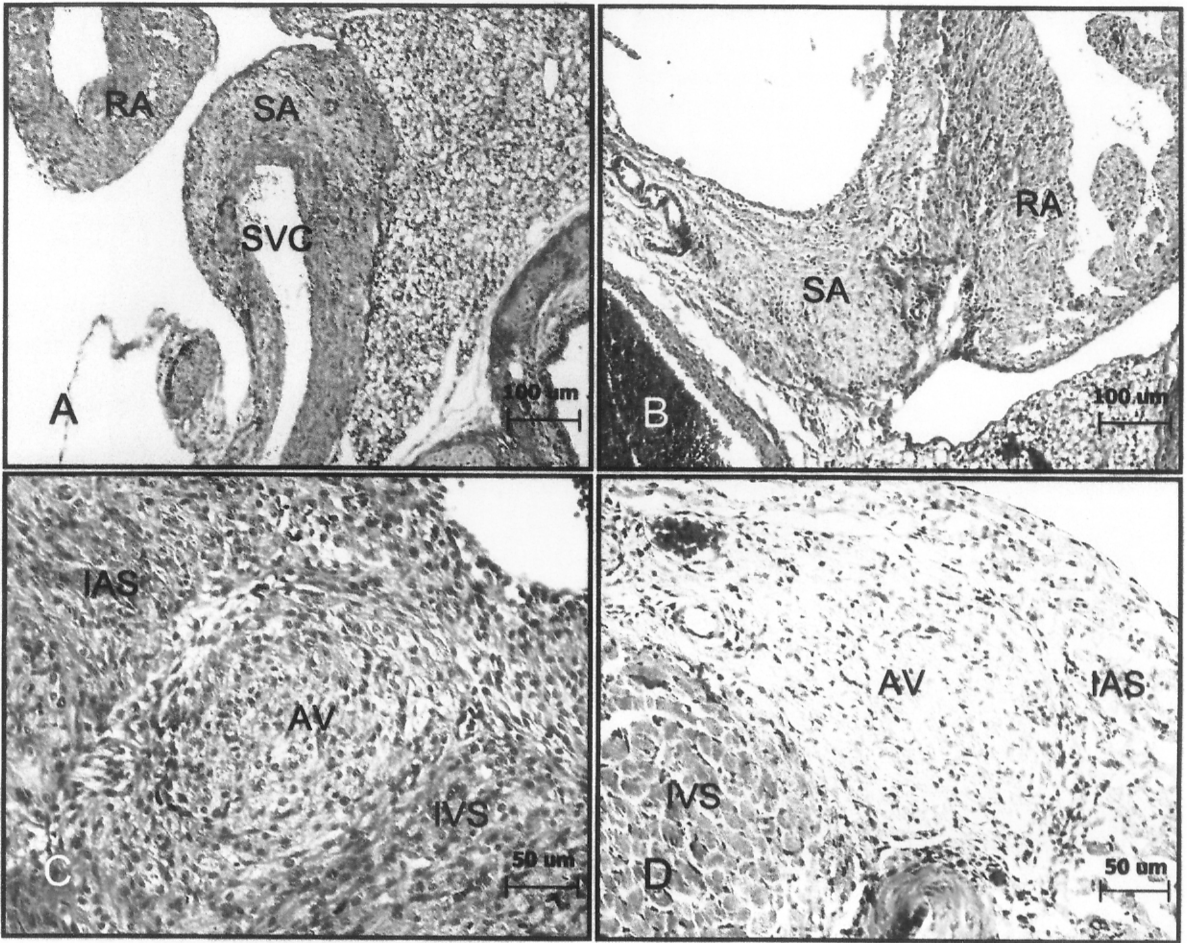

Fig.1 A-D Micrographs of Masson Trichrome stained sections showing sinoatrial node (SA) in (A) 2-day SD (B) 2-week SD, and atrioventricular node (AV) in (C) 2-day SD (D) 2-week SD rat hearts. SVC: superior vena cava, RA: right atrium, IVS: interventricular septum, IAS: interatrial septum

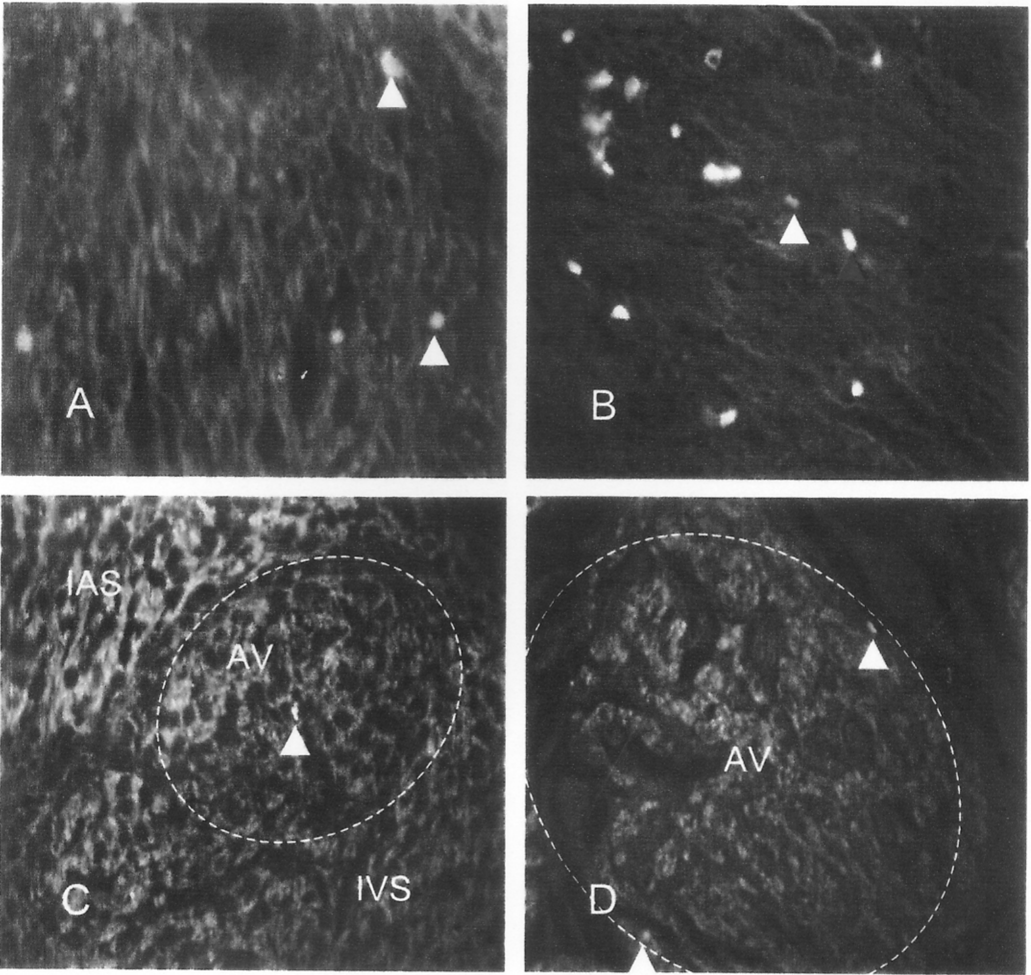

Fig. 2A-D Micrographs showing Fluorescein-FragEL^{TM} labeled nuclei (white arrows) in the sinoatrial node (SA) AV node of (A) 2-dayTG (B) 1-week SD and the AV node of (C) 2-day TG (D) 2-week TG rat hearts. X 400, blue arrows: red blood cells IVS: interventricular septum, IAS: interatrial septum

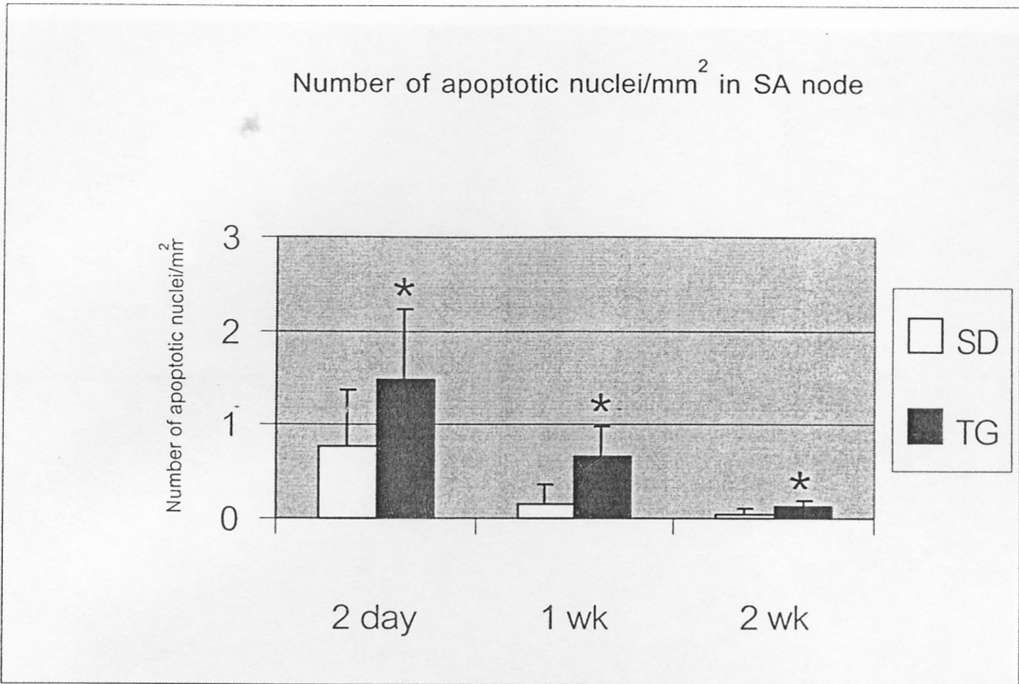

Fig. 3 Bar chart showing number of apoptotic nuclei/mm^2 in the SA node of 2-day, 1-week and 2-week SD and TG rats; data are expressed as mean± SEM, Student's T-test, *P<0.05, N=6

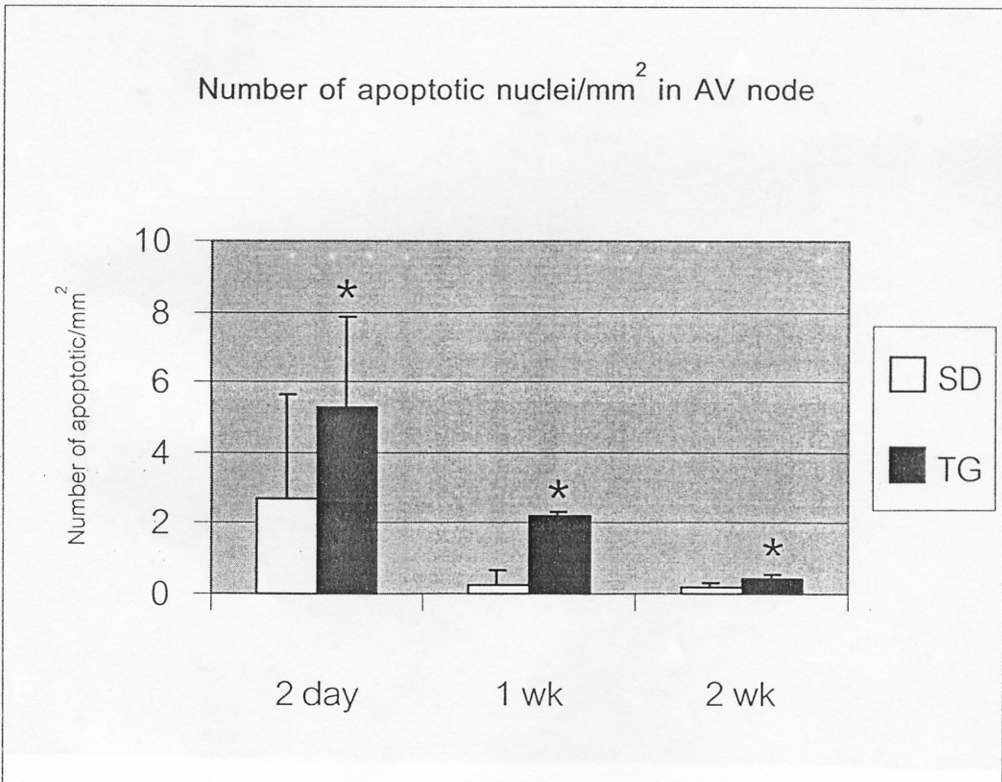

Fig. 4 Bar chart showing number of apoptotic nuclei /mm^2 in the AV node of 2-day, 1-week and 2-week SD and TG rats; data are expressed as mean± SEM, Student's T-test *P<0.05, N=6

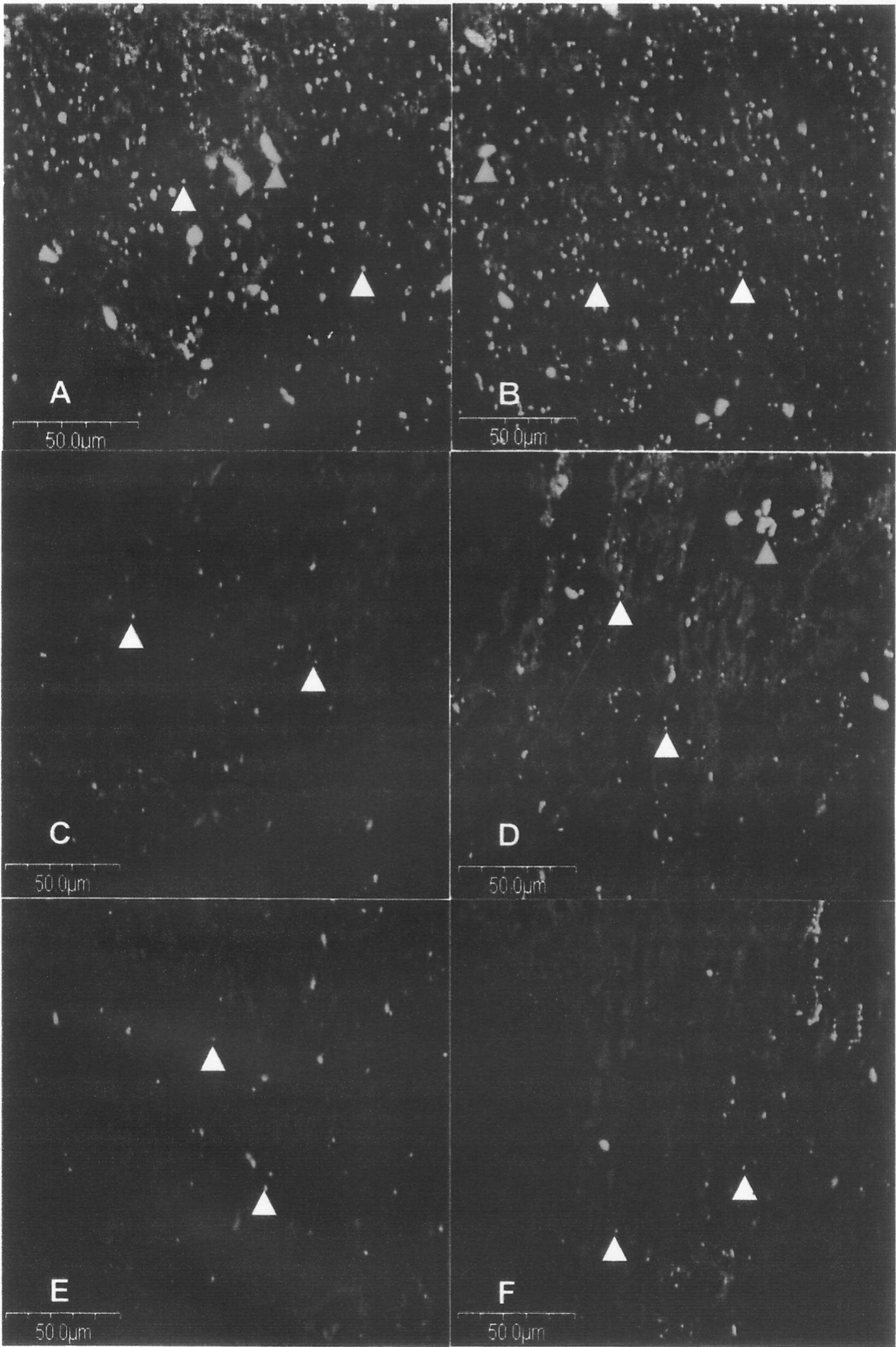

Fig. 5A-F Confocal micrographs showing the distribution of AT_1 receptors (white arrows) in sinoatrial node (SA) of (A) 2-day SD (B) 2-day TG (C) 1-week SD (D) 1-week TG (E) 2-week SD (F) 2-week TG rat hearts. blue arrows: red blood cells

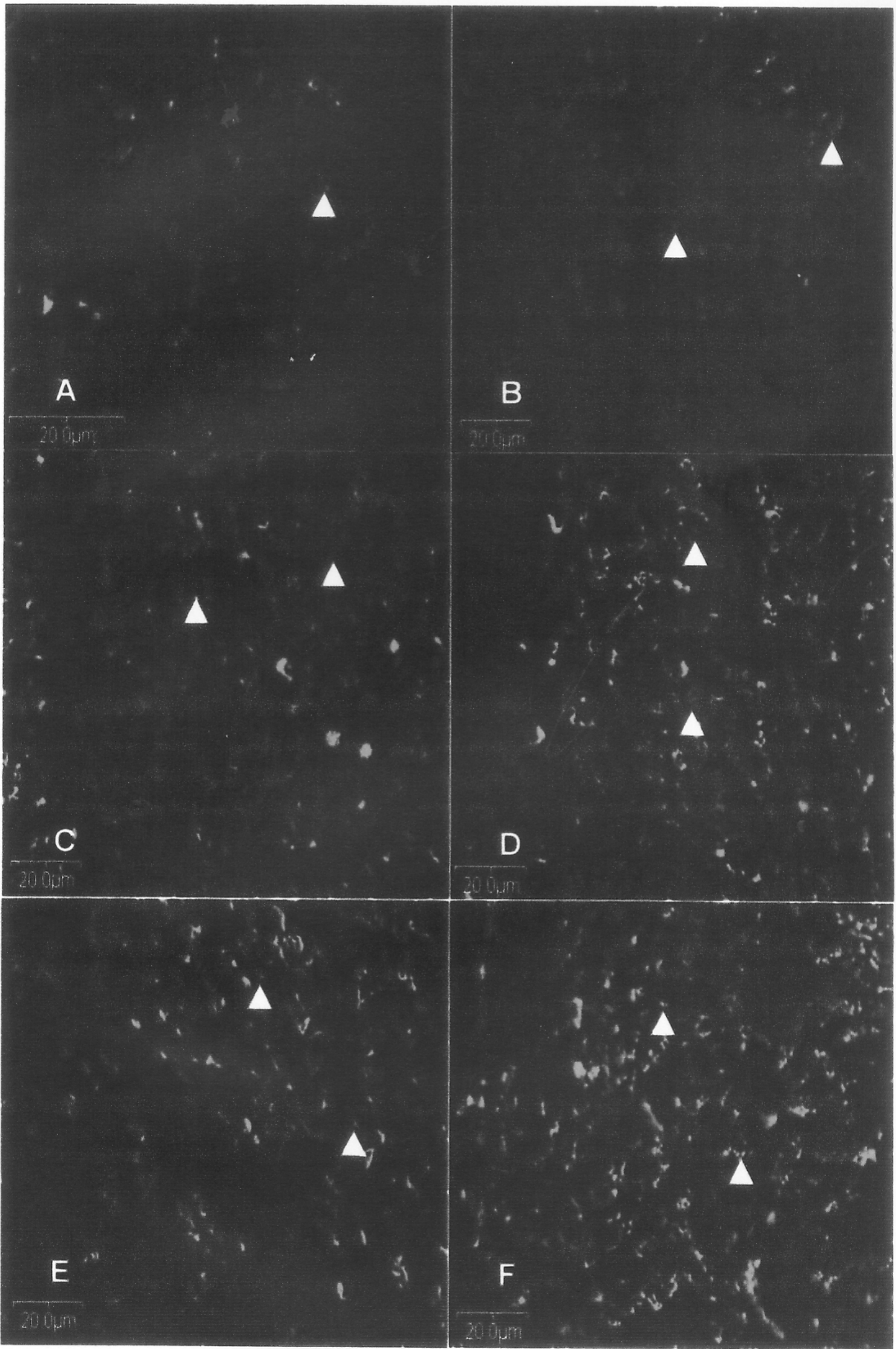

Fig. 6A-F Confocal micrographs showing the distribution of AT_1 receptors (white arrows) in atrioventricular node (AV) of (A) 2-day SD (B) 2-day TG (C) 1-week SD (D) 1-week TG (E) 2-week SD (F) 2-week TG rat hearts.

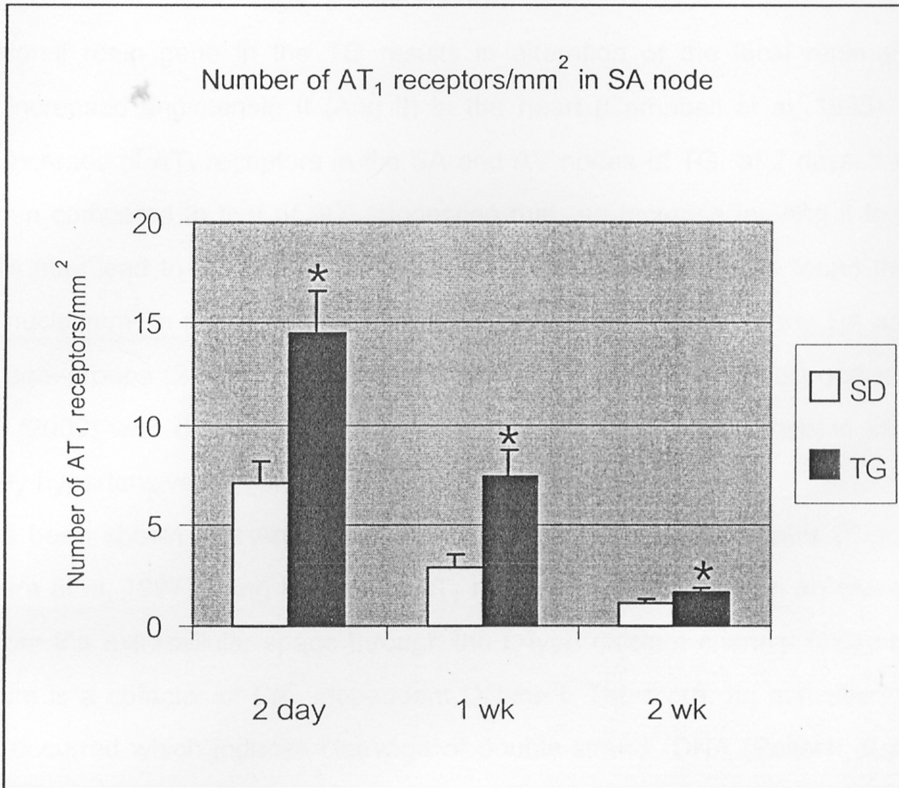

Fig. 7 Bar chart showing number of AT_1 receptors/mm^2 in the SA node of 2-day, 1-week and 2-week SD and TG rats; data are expressed as mean± SEM, Student's T-test *P<0.05, N=6

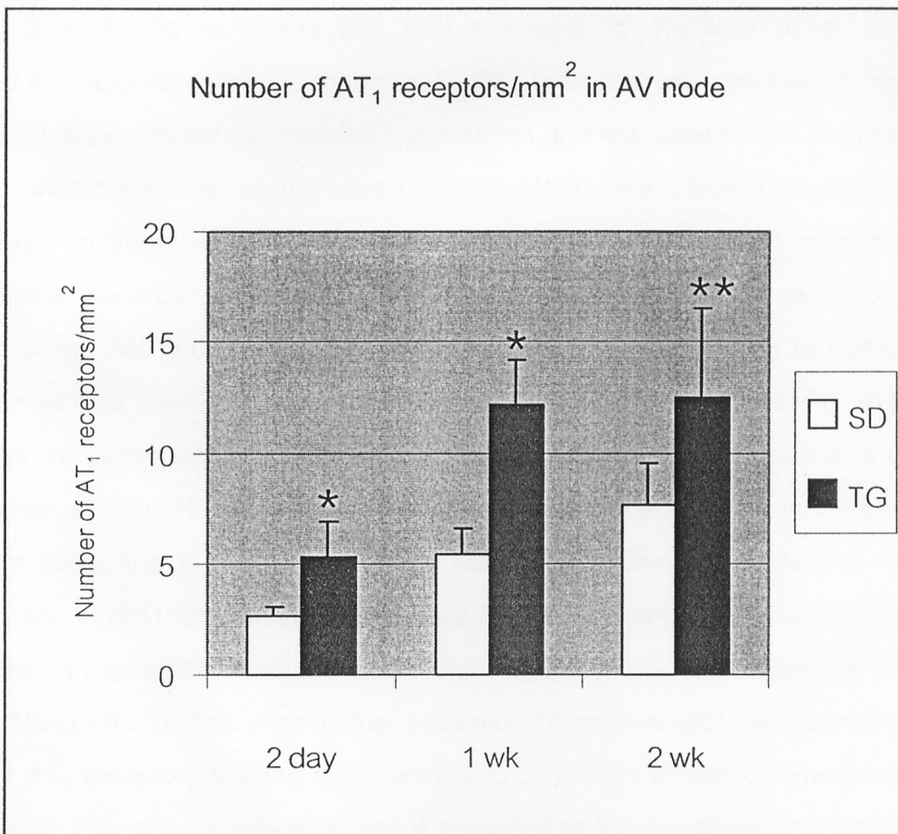

Fig. 8 Bar chart showing number of AT_1 receptors/mm^2 in the AV node of 2-day, 1-week and 2-week SD and TG rats; data are expressed as mean± SEM, Student's T-test *P<0.05, **P<0.01,N=6

Discussion

Additional renin gene in the TG results in alteration of the local renin-angiotensin system, i.e. increased angiotensin II (Ang II) in the heart (Campbell et al, 1995). Our data showed an increase of AT_1 receptors in the SA and AV nodes of TG at 2 days, 1 week and 2 weeks (when compared to that of SD) suggesting that an increase in Ang II in heart and AT_1 receptors may lead to the higher apoptosis. In the present study, we found the number of apoptotic nuclei/mm^2 in the TG to be more than that of the SD in both the SA and the AV nodes in all age–groups (2 days, 1 week and 2 weeks). The results correspond to the work of Lie et al., (2000) who demonstrated that there is an increased apoptosis in the heart of spontaneously hypertensive rat (SHR).

It has been shown that Ang II via AT_1 receptors mediates apoptosis (Cigolar et. al, 1997; Kajstura et al, 1997). Ang II binds to AT_1 receptors, what leads to an elevated influx of calcium from the extracellular space through the L-type calcium channel (Dosemea et al., 1988). Calcium is a cofactor of Ca^{2+} dependent DNase I. Therefore, an activation of DNase enzyme has occurred which induces cleavage of double-strand DNA (Peitsch et al., 1993). Thus, Ang II via AT_1 receptors may mediate apoptosis in the SA and the AV nodes similar to cardiomyocyte.

We found that the apoptosis in the SA and the AV nodes was reduced with maturation. This finding is in the line with the work of Kajstura et al, (1995) who demonstrated that apoptosis occurs immediately after birth and was reduced at day 11, after which the apoptosis could not be detected. In addition, the few weeks after birth is the major period when morphogenesis of the conduction system take place (James, 1994), and apoptosis plays an important role. This might explain the reduction of apoptosis after 2 weeks. Excess apoptosis has been demonstrated to be a possible cause of abnormal development of the AV node, SA node and internodal pathway, leading to complete heart block and arrhythmia (Jame et al, 1996). Therefore, apoptosis is possibly the principal mechanism for the pathogenesis of arrhythmia in human infants associated with an abnormal renin-angiotensin system. However, apoptosis may not explain arrhythmia in the elderly.

On its own, Ang II via AT_1 receptors has been shown to be directly involved in arrhythmia. Kass et al (1981) have demonstrated that stimulation of AT_1 receptors on the SA and AV nodes can generate spontaneous electrical activity, or alter responses to electrical stimulation. Mello (2001) has shown that activation of renin–angiotensin system results in stimulation of AT_1 receptors leading to an activation of protein kinase C. Phosphorylation of junctional protein then occurs, which causes a reduction of the junctional conductance. As a

consequence, the conduction velocity is decreased or conduction is blocked, leading to reentrant rhythms.

We have shown that the number of AT_1 receptors in the SA node in both the SD and TG is reduced from 2 days to 2 weeks whereas in the AV node, the number of AT_1 receptors is increased. According to the work of Sechi et al (1990), who demonstrated that at 10 and 14 weeks, the density of the binding sites of AT_1 receptors in AV node is high, whereas it is low in the SA node. This data confirm that AT_1 receptors in the AV node increase with maturation, but in the SA node it decreases with maturation, suggesting that at a very young age AT_1 receptors may be involved in SA node function rather than AV node, whereas in aging animals they may act on the AV node rather than on the SA node. The cause of AT_1 receptors alteration in the SA and AV node associated with age requires further investigation.

Our data has demonstrated that there are more AT_1 receptors in both the SA and the AV nodes in the TG rats in all age-groups than in the SD rats. The overexpression of AT_1 receptors has been shown to be a major cause in mediating bradycardia associated with heart block, producing a lethal phenotype within the first week after birth in MHC–AT_1 transgenic mice (Hein et al., 1997). Treatment with a selective AT_1 antagonist like CV-11974 has been shown to prevent arrhythmias in wild-type mice (Harade et al, 1998). Recently, AT_1 receptors antagonist Losartan appeared to reduce mortality in older patients with chronic congestive heart failure by 40%. This was due to fewer sudden cardiac deaths, suggesting that complete blockade of AT_1 receptor may results in preventing arrhythmia (Pitt et al., 1997). This supports the role of Ang II in associated with inducing arrhythmia.

Conclusion

From the present study, we suggest that Ang II via AT_1 receptors may induce arrhythmia. However, this may not be due to its mediating role in apoptosis, instead the reduction of the junctional conduction or reentrant rhythms may be a possible cause. Arrhythmia associated with the alteration of angiotensin when animals getting older occurs more often at the AV node than at the SA node.

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