# Expression of Cardiac Renin and its Modulation by Stress in Normotensive and Hypertensive Rats

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**Abstract.** Renin is the rate-limiting step in angiotensin II production Existence of the cardiac renin is still ambiguous in healthy animals, although there is evi dence that under some pathological conditions the heart might express mRNA for renin Therefore, the aim of the present study was to (i) detect the renin gene expression in the whole rat heart, ventricles, atria and in isolated and purified myocytes, (ii) determine the effect of stress on renin mRNA and protein levels, and (iii) compare the response of renin gene expression to stress in normoten sive and spontaneously hypertensive rats Renin mRNA was determined by reverse transcription and polymerase chain reaction and quantified relatively to $\beta$ actin and glyceraldehyde-3 phosphate dehydrogenase Protein message was detected by monoclonal antibody against renin Renin mRNA was found in all parts of the heart and in myocytes Renin protein was found in the heart ventricles and atria, but not in cardiomyocytes Immobilization stress affected renin on both, the mRNA and the protein level The effect of stress was observed in the hearts of normoten sive, but not in genetically hypertensive rats Thus, renin might be involved in the development of the pathophysiological state in rat heart

**Key words:** Renin — Myocytes — Cardiac — Stress

## Introduction

The renin angiotensin system (RAS) is a metabolic pathway producing biologically active angiotensin II (AII), which is known to be a potent vasoconstrictor Circulating RAS is deeply involved in the regulation of blood pressure and in fluid and electrolyte homeostasis Renin is a key enzyme of the renin-angiotensin system, and changes in the biosynthesis and renal secretion of renin are normally the prime and apparently the sole determinants of a change in plasma AII formation Levels of the circulating renin are finely regulated and subjected to physiological adjustment

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(von Lutterotti et al 1994) Although kidneys are the primary and apparently the sole source of active renin in the circulation, renin is also expressed in several other tissues However, the existence of the cardiac renin is still equivocal

Several laboratories have reported that under normal physiological conditions cardiac renin mRNA levels are undetectable or extremely low (Ekker et al 1989, Iwai and Inagami 1992, Danser et al 1993) However, under pathological conditions the expression of renin might be induced Such expression was reported in the rat ventricle after myocardial infarction (Passier et al 1996) and after low sodium diet and treatment with the ACE inhibitor enalapril in the rat atrium (Lou et al 1993) On the other hand, several other laboratories detected renin mRNA in the heart of rats (Dzau and Re 1987, Paul et al 1988, Suzuki et al 1988), mice (Field et al 1984), and humans (Danser et al 1997)

In the present study we focused on two aspects arising from the uncertainty about the existence of cardiac renin We measured expression of the renin mRNA not only in the heart and/or its individual parts, but also in isolated and purified myocytes, to avoid other cell contamination Immobilization stress is known to affect several processes in various tissues, e g induction of enzyme activity and/or expression of components participating in the production of catecholamines, which results in a rapid increase of catecholamines (Kvetnansky and Mikulaj 1970, Kvetnansky and Sabban 1993), Ca-transport in the kidney (Hudecova et al 1996) or plasma renin activity (Jindra and Kvetnansky 1982, Krizanova et al 1996) Based on the observation of other authors that renin might be induced under the pathological conditions (Field et al 1984, Lou et al 1993), we also studied the effect of immobilization stress on the cardiac renin in normotensive Wistar Kyoto (WKY) and Sprague-Dawley rats and in spontaneously hypertensive rats (SHR)

The present study supports the opinion about the cardiac renin and reports for the first time that immobilization stress increases cardiac renin on both, the mRNA and the protein level in normotensive Sprague-Dawley and Wistar-Kyoto rats Cardiac renin in spontaneously hypertensive rats is not modulated by immobilization stress

## Materials and Methods

### *Animals*

The protocol used was approved by the Animal Care Committee of the Slovak Academy of Sciences and the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia Male, specific pathogen free, Sprague-Dawley rats (280 350 g, three months old) were obtained from Charles River Laboratories (Sulzfeld, Germany) Adult spontaneously hypertensive rats and Wistar-Kyoto rats were obtained from the same source Before initiation of the experimental procedures, the animals were housed 3 4 per cage for at least 7 days under light-controlled conditions (light on from 6 AM to 6 PM) at a room temperature of 23 ± 2°C Food and water were available *ad libitum* All experiments were done between 8 AM and 2 PM

*Immobilization experiments*

Immobilization (IMO) was carried out by taping the four limbs of each rat to specially prepared metal mounts attached to a board (Kvetnansky and Mikulaj 1970). The animals were sacrificed by decapitation immediately after 120 min of IMO or 3 hours after having been returned to their home cages. Control (non-stressed) rats were decapitated directly after removal from their home cages. Repeated stress was done by immobilizing animals seven and/or forty-two times for 2 hours daily. Immediately after decapitation the hearts were dissected, individual parts of the heart separated, frozen in liquid nitrogen, and stored at −70 °C for later analysis. Each experimental group consisted of 6–12 animals.

*Determination of the plasma renin activity (PRA) and the kidney renin activity (KRA)*

PRA was determined using [$^{125}$I] Angio I RIA kit (Immunotech, Czech Republic) according to manufacturer's protocol. PRA detection was performed indirectly by determining *in vitro* generated angiotensin I. This kit has the cross-reactivity with angiotensin II smaller than 0.001%. RIA inter-assay variability was approximately 11% and the intra-assay variability was approximately 6%. Minimal detectable concentration of angiotensin I was 0.007 ng/ml. Plasma was prepared by centrifugation of blood samples for 30 min at 3000 × *g*.

KRA was measured by the same kit after the addition of the angiotensinogen substrate. The substrate was prepared from bilaterally nephrectomized male rats. Twenty-four hours after the surgery, the animals were sacrificed by decapitation and the blood was collected into vials containing 0.5 mol/l EDTA, pH 8.0 in an approximate amount of 1/10$^{th}$ of the total blood volume.

*Myocyte preparation*

Myocytes were obtained by enzymatic dispersion from the rat hearts, according to the procedure of Zahradník and Zahradníková (1989) with the following modification. The cells were isolated in Ca-free KB solution (106 mmol/l methanesulphonic acid, 106 mmol/l potassium hydroxide, 39 mmol/l potassium chloride, 2.4 mmol/l magnesium sulphate, 8 mmol/l potassium hydrophosphate, 1 mmol/l EGTA, 22 mmol/l taurin, 10 mmol/l HEPES, 10 mmol/l glucose) and afterwards centrifuged on a discontinuous Percoll gradient (5 ml – 30%, 5 ml – 37.5%, 5 ml – 43.5%, 5ml – 50% Percoll, density 1.130 ± 0.005 g/l) at 500 × *g* for 15–20 min. After the isopycnic centrifugation the cell fractions were harvested by aspiration and each fraction was checked for viability on Nikon 108 microscope.

*RNA preparation*

RNA was isolated from rat hearts according to the procedure of Chomczynski and Sacchi (1987) using guanidine isothiocyanate (Fisher Scientific, USA) and phenol-chloroform extraction. Briefly, the cardiac tissue or myocytes were homogenized in guanosine – thiocyanate with 2-mercaptoethanol, phenol, sodium acetate and a mixture of chloroform : isoamylalcohol (24:1 v/v) and mixed thoroughly. After 15

minutes of the incubation on ice, the homogenate was centrifuged for 20 minutes (12,000 rpm, 4 °C) To the aqueous phase an equal volume of isopropanol was added and kept at −20 °C for 1 hour After the precipitation, the mixture was centrifuged for 20 minutes (12,000 rpm, 4 °C) The RNA pellet was dissolved in RNase-free water and extracted twice with phenol chloroform (1 1 v/v) The last aqueous RNA-phase was mixed with $1/10^{th}$ volume of 3 mol/l sodium acetate and twofold volume of 96% ethanol RNA was precipitated overnight at −20 °C Afterwards, the RNA pellet was washed with 75% ethanol and dissolved in RNase-free water The concentration and purity of RNA were determined spectrophotometrically on Shimadzu UV-3000 (Kyoto, Japan)

*Relative quantification of mRNA levels by RT-PCR*

Reverse transcription was done using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech), with pd(N)$_6$ primer For each RT-PCR analysis, 5 $\mu$g of the total RNA was used The number of cycles was determined for each kind of RT-PCR separately, testing 15, 20, 25, 30, 35, 37 and 40 cycles By this procedure it was verified that under the described conditions the PCR amplification of each fragment was still within the linear range Control reactions were performed omitting reverse transcription to determine whether RNA samples were contaminated with DNA Specific PCR for the renin was performed afterwards using RR1 (5′-TCT CAG CAA CAT GGA CTA TGT GC-36′) and RR2 (5′-TTA GCG GGC CAA GGC GAA CC-346′) primers designed according to Pieruzzi et al (1995) giving 190 bp fragment The PCR program included 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and polymerization at 72 °C for 1 min As a control for quantitative evaluation of PCR, $\beta$-actin primers (BA1 5′-AGT GTG ACG TTG ACA-3′, BA2 5′-GAC TGA TCG TAC TCC TGC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (GPH1 5′-AGA TCC ACA ACG GAT ACA TT-3′, GPH2 5′-TCC CTC AAG ATT GTC AGC AA-3′) were used to amplify the 240 bp fragment for $\beta$-actin (Lou et al 1995), after denaturation at 94 °C for 5 min and 30 cycles of PCR at 94 °C, 55 °C and 72 °C for 1 min each, and the 309 bp fragment for GAPDH (Terada et al 1993) from each first strand sample, after denaturation at 94 °C for 5 min, 30 cycles of PCR at 94 °C, 60 °C for 1 min and 72 °C for 3 min each PCR products were analyzed on 2% agarose gels The intensities of the individual bands were measured by a Kodak photo-camera, quantified using IMAGE software and compared to both, $\beta$-actin and GAPDH

*Polyacrylamide electrophoresis of the proteins (PAGE-SDS)*

PAGE was performed according to the procedure of Laemli (1970) on 6% polyacrylamide gels The protein extracts were prepared by tissue and/or cell homogenization and/or lysis, using an extraction buffer (20 mmol/l Tris-HCl pH 7 0, 0 3 mol/l sucrose, 5 mmol/l phenyl methyl sulfonyl fluoride, 5 mmol/l iodacetamide and 5 mmol/l benzamidine) to the final amount of 100 mg tissue in 1 ml extraction buffer 20 $\mu$g of the protein extracts were mixed with a loading buffer (10 mmol/l Tris HCl, 2% sodium dodecyl sulphate, 20% glycerin) with 5% 2-mercaptoethanol

The mixture was boiled for 5 minutes and loaded to gels The electrophoretic separation ran at 70 V

*ECL-Western Blotting*

After PAGE, proteins were transferred to the HYBOND C+ nitrocellulose membrane (Amersham, UK) and hybridized by mouse monoclonal antibody against rat renin (SWant, Switzerland) This antibody was developed against mouse submandibular gland renin but it also recognizes rat renin in immunohistochemistry, immunoblots and dot-immunobinding assays Nitrocellulose membranes were blocked in 5% dried milk without fat, dissolved in 1xTBST buffer with 0 1% Tween 20 for one hour or overnight Afterwards, the membranes were incubated with renin antibody (diluted 1 2000) for 1 hour The membranes were washed 3-times in 1xTBST and incubated with a secondary antimouse antibody (1 10,000) for 1 hour Detection was done with ECL (Amersham, UK) on RTG films Quantification of the individual bands was done using IMAGE software

*Statistical analyses*

Each value is an average from at least 6 animals Results are presented as means ± S E M Statistical differences among groups were determined by one way analysis of variance (ANOVA) Statistical significance was defined as $p < 0\ 05$ and $p$ values were adjusted for multiple comparisons by the Bonferroni method (Instat, GraphPad Software, USA)

## Results

*Cardiac renin-angiotensin system in myocytes and the heart*

In order to detect the renin message in cardiac myocytes, we measured the gene expression by RT-PCR (Fig 1) The quantity of the expression was determined relative to the expression of a housekeeper GAPDH (not shown) We used kidney (KR) as a positive control, where renin is expressed in high quantities We found the renin mRNA also in the isolated and purified cardiac myocytes, although in lesser

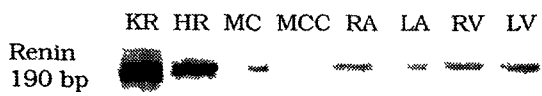


**Figure 1.** Renin mRNA expression in the whole heart (HR), isolated and purified myocytes (MC), left (LV) and right (RV) ventricles, left (LA) and right (RA) atria mRNA levels in the kidney (KR) were estimated as the positive control To determine that the signal in the myocytes is due to mRNA expression of the renin gene and not due to genomic DNA contamination, a negative control (MCC) was performed, omitting reverse transcription Quantification was done relative to $\beta$-actin and GAPDH (not shown) The results shown are typical results from 7–8 animals

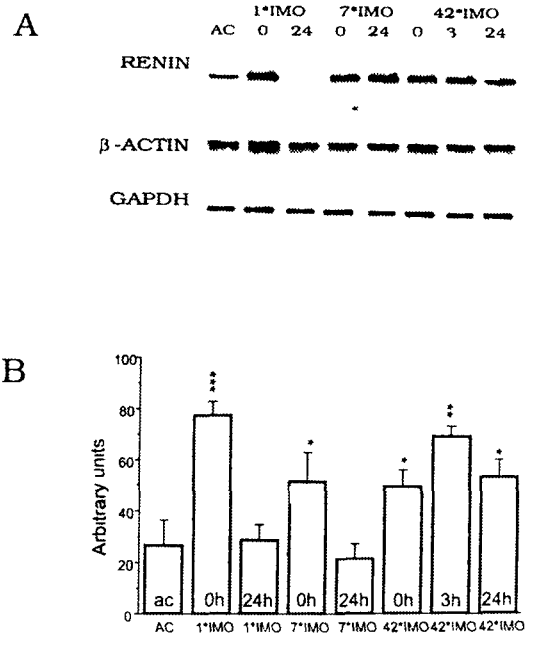


**Figure 2.** The effect of immobilization stress on cardiac renin expression (*A*) and protein levels (*B*) in Sprague-Dawley rats Renin mRNA was detected by RT-PCR and quantified relative to two housekeepers, $\beta$-actin and GAPDH Renin protein was measured by Western blot analysis with subsequent immunodetection done by monoclonal antibody against rat renin Each value is expressed as mean ± S E M , and represents an average from 7–8 measurements Significance was calculated by ANOVA, *** – $p < 0\ 001$, ** – $p < 0\ 01$, * $p < 0\ 05$ With respect to both, renin mRNA and renin protein, immobilization resulted in increases at the end of a single immobilization with subsequent decrease to control levels after 24-hour rest After 7 immobilizations, renin mRNA remained increased even after 24-hour rest, whereas protein message decreased similarly as after a single immobilization After 42 immobilizations both mRNA and protein remained increased

quantities than in the whole heart The band observed for myocytes (MC) was due to the presence of mRNA for renin rather than an artifact from residual genomic DNA, since upon elimination of the reverse transcription no band appeared for the myocytes (MCC) Western blot analysis with subsequent monoclonal antibody binding did not reveal the presence of renin protein in the myocytes, probably due to a very small amount of renin, which was under the detection limit of this procedure

*The effect of immobilization stress on local renin in Sprague-Dawley rats*

All further experiments were performed on whole hearts Immobilization stress increased both, renin mRNA (Fig 2*A*) relative to $\beta$-actin and GAPDH, and the

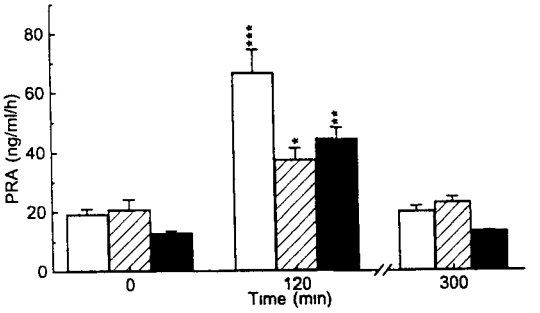


**Figure 3.** The effect of single and repeated immobilization stress on plasma renin activity (PRA) The peak of PRA appeared after 30 min of immobilization with a subsequent decline in PRA after 120 min of immobilization stress The profiles of PRA after single (white columns), 7-times immobilized (striped columns) and immobilization for 42-times (black columns) did not show significant differences in profile Each value is expressed as mean ± S E M , and is an average from 7 8 measurements Significance was calculated by ANOVA *** $p < 0\ 001$, ** $p < 0\ 01$, * - $p < 0\ 05$

protein message of renin (Fig 2*B*) to different extend according to the number of immobilizations After 2 hours of stress stimulus, the renin mRNA and protein increased rapidly After 24 hours of rest, the levels of renin mRNA and protein message declined to the baseline Similar results were obtained after seven immo bilizations for protein message, while mRNA for renin remained increased even after 24-hours rest Significant adaptation in both compounds was noticed after 42 immobilizations

*The effect of immobilization stress on plasma renin activity (PRA) in Sprague-Dawley rats*

These experiments were done to compare the response of the circulating renin (PRA) to immobilization stress with the response of the cardiac renin PRA reached a maximum after 30 min of immobilization (Fig 3) A comparison of the PRA profiles after single (hollow columns), 7-times repeated (hatched columns) and 42-times repeated (black columns) immobilization did not show any significant differences (Fig 3), we therefore assume that neither 42-times repeated immobilization caused the adaptation of PRA to this stimuli

*Response of spontaneously hypertensive rats to immobilization stress*

Expression of cardiac renin was much higher in SHR than in WKY, compared to the housekeeper GAPDH (Fig 4) This result nicely corresponds to the amounts of renin protein in SHR and WKY rat hearts (Fig 5AC)

A significant difference in the response to stress was observed in SHR compared to Wistar-Kyoto rats (Fig 5) While in the hearts of WKY rats the amounts of renin were significantly increased even after a single immobilization (Fig 5, hatched

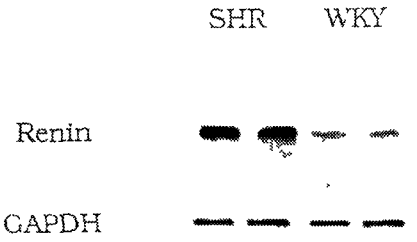


**Figure 4.** Renin expression in the hearts of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) Renin expression was quantified relative to the housekeeper glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

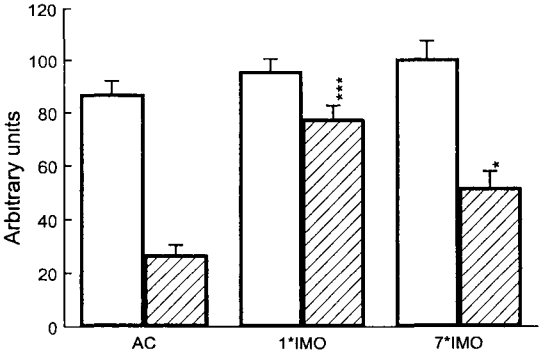


**Figure 5.** The effect of immobilization stress on the cardiac renin protein in spontaneously hypertensive rats (hollow columns), compared to Wistar-Kyoto rats (striped columns) Renin protein was measured by Western blot analysis with subsequent immunodetection done by monoclonal antibody against rat renin Each value is expressed as mean ± S E M , and represents an average from 7-8 measurements Significance was calculated by ANOVA, *** – $p < 0\ 001$, * – $p < 0\ 05$ In comparison to Wistar-Kyoto rats, no significant difference in SHR was observed between control and immobilized animals in renin protein

columns), in the hearts of SHR rats the levels of renin remained at the same value after single and/or repeated immobilization (Fig. 5, hollow columns). In SHR rats, renin mRNA was not significantly changed by the stress stimuli (not shown).

## Discussion

The cardiac renin-angiotensin system can have detrimental effects on heart function and is involved in several pathophysiological conditions of the heart. Since a funda-

mental requirement for demonstrating the local synthesis of a protein is evidence for gene expression within the tissue, we checked mRNA for renin in the individual parts of the rat heart and/or cardiac myocytes. Despite some doubts about the existence of cardiac renin (Field et al. 1984; Danser et al. 1993), we successfully amplified the renin signal by RT-PCR not only from the entire heart, isolated ventricles and atria, but also from isolated and purified myocytes. We observed a clear, although weak signal for renin mRNA in myocytes. The origin of this signal was not from genomic DNA but from renin mRNA. Since the relative renin signal from the whole heart was much stronger with respect to both housekeepers than that from myocytes, we suggest that the bulk of cardiac renin originates from cells other than myocytes, e.g. fibroblasts.

As mentioned previously, the cardiac renin-angiotensin system might be involved in several pathological conditions of the cardiovascular system. Stress is known to participate in several pathophysiological conditions. RAS is partially under the control of the sympathoadrenal system (Johnson et al. 1979), which is also activated by many stressors (Kvetnansky et al. 1978). PRA was shown to be increased by stress (Jindra et al. 1980; Krizanova et al. 1996). Therefore, we were interested in the response of cardiac renin due to single, repeated and prolonged stress stimuli. We have found that single immobilization reversibly increased both the mRNA and protein for renin. After repeated immobilization we saw an adaptation in the expression of renin mRNA, while the increase in renin protein was still reversible. The difference between renin mRNA expression and renin protein levels after repeated immobilization could be explained by some extracardiac factors. Adaptation occurred in both, mRNA and protein for renin after the prolonged immobilization. These results differ from the behavior of circulating renin determining the PRA activity, where neither the prolonged immobilization did cause the adaptation.

In spontaneously hypertensive rats, structural changes in arterial walls are associated with high blood pressure, consisting in an increase in wall thickness in large arteries (Olivetti et al. 1980). Angiotensin II has been shown to play an important role in the control of blood pressure and vascular structure, because ACE inhibitors have a persistent inhibitory effect on SHR whereas other antihypertensive drugs do not (Christensen et al. 1989). It has already been shown that the cardiac renin-angiotensin system is involved in the altered cardiac gene expression in SHR (Okura et al. 1991). Therefore, the cardiac renin-angiotensin system may be of a special interest in this type of rats. Surprisingly, cardiac renin did not respond to immobilization stimuli, either on the mRNA, or on the protein level. However, the renin mRNA was much more abundant in SHR compared to Sprague-Dawley and Wistar-Kyoto rats.

The physiological relevance of the increased levels of cardiac renin under some pathophysiological stimuli remains to be elucidated. However, since renin is a key enzyme of the renin-angiotensin pathway, it is highly probable that elevated levels of this enzyme will result in increased levels of angiotensin II in the rat heart. Angiotensin II is known to increase the intracellular calcium concentration in the

heart (Krizanova et al. 1997), rat portal vein myocytes (Morel et al 1996), and in many other cells. Therefore, the enhanced renin levels might result in an elevation of intracellular calcium levels in the myocardium. Evidence has however to be brought for this assumption.

In summary, in this work we confirmed the existence of renin mRNA not only in the heart tissue and/or in its individual parts, but also in isolated and purified myocytes. Also, this paper is the first to report that immobilization stress increases both, renin mRNA and renin protein in normotensive Sprague-Dawley rats However, in spontaneously hypertensive rats no such increase was observed These results suggest that in normotensive Sprague-Dawley rats stress is handled by a different mechanism than in spontaneously hypertensive rats

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