ENHANCED EXPRESSION OF MINERALOCORTICOID RECEPTORS IN THE HEART AFTER THE MYOCARDIAL INFARCT IN RATS

Increasing evidence suggests that enhanced stimulation of the heart and kidney by mineralocorticoids plays a significant role in the development of the post-infarct cardiac failure. Because increased synthesis of mineralocorticoid receptors (MR) is one of the putative factors determining the pathogenic effects of mineralocorticoids, we decided to determine whether the myocardial infarct results in an enhanced expression of MR mRNA and MR protein. To this end, male Sprague-Dawley rats were subjected either to ligation of the left coronary artery or to sham surgery. After four weeks, expressions of MR mRNA and MR protein were evaluated in both groups of rats in the left (LV) and right (RV) ventricle walls, and in the renal cortex and renal medulla by means of semiquantitative PCR and Western blotting methods. Coronary ligation resulted in the myocardial infarction encompassing 30.2% ± 1.9% (range 23-40%) of the left ventricle wall. In the infarcted rats, expression of MR mRNA was significantly greater than in the sham-operated rats, both in the LV (P<0.02) and in the RV (P<0.005). In the left but not in the right ventricle, increased MR mRNA expression was associated with a significant increase in expression of MR protein (P<0.001). In the renal cortex and renal medulla, MR mRNA and MR protein expression in the infarcted and the sham-operated rats did not differ. The study reveals that during the post-infarct state expression of MR mRNA is elevated in both cardiac ventricles while expression of MR mRNA protein is increased only in the left ventricle. The results suggest that the enhanced expression of mineralocorticoid receptors may contribute to enhanced effects of mineralocorticoids in the heart during the post-infarct state.

Key words: heart infarct, heart failure, MR mRNA, cardiac hypertrophy
INTRODUCTION

It is now well established that mineralocorticoids play significant role in regulation of sodium and potassium balance (1, 2). Recently, the evidence has been provided for synthesis of aldosterone and presence of MR and MR mRNA in non-classical locations, such as the cardiovascular system and the brain (3 - 8). In addition, significant changes in expression of MR mRNA and protein in the extrarenal locations has been found in certain models of hypertension (7, 9). Recently, it has been postulated that the altered stimulation of the extrarenal mineralocorticoid receptors may play an important role in cardiovascular pathology (10 - 13). This poses a question whether the negative effects of mineralocorticoids in the cardiovascular diseases may result from an enhanced transcription of MR gene and an increased abundance of MR protein. The specific purpose of the present study was to determine expression of mineralocorticoid receptors during the post-infarct state in the heart and the kidney - the two target organs of mineralocorticoids. Thus far changes in expression of MR after the myocardial infarction has not been determined under controlled experimental conditions. Previously, enhanced expression of MR mRNA and MR receptors in the heart was found in the patients suffering from the congestive heart failure (14). However, in the latter study the pathogenesis of cardiac failure was not uniform and the influence of pharmacological treatment or other factors not directly associated with the cardiac failure could not be excluded. The rationale to determine MR mRNA and protein expression in the kidney was based on several lines of evidence showing significant changes in function of the kidney during the post-infarct state. The myocardial infarct results in activation of several neuroendocrine systems which have influence on synthesis of mineralocorticoid receptors in the kidney (stimulation of renal sympathetic fibers, and renin-angiotensin-aldosterone system, elevation of circulating vasopressin, natriuretic peptides and cytokines (13, 15 - 19). To avoid nonspecific effects of anesthesia and surgical trauma and to evaluate prolonged impact of the post-infarct state on expression of mineralocorticoid receptors measurements of MR mRNA and MR protein were performed on tissue samples harvested four weeks after ligation of the coronary artery. Some preliminary data of this study have been reported at the scientific meeting (20).

MATERIALS AND METHODS

Animals and surgical procedures

The study was performed on male Sprague Dawley rats (SPRD/Mol/Lod) that were bred in the Department of Animal Breeding of the Medical University of Warsaw. The rats were maintained on 12-h light/12-h dark rhythm in the room with regulated temperature (range 22 - 25 °C) and had free access to water and rodent dry pellet diet containing 0.3% of NaCl. The experimental protocol was approved by the Ethical Committee on the Animal Research of the Medical University of Warsaw.
At the beginning of the study 10 weeks old rats were divided into two groups. One group was subjected to myocardial infarction (MI) according to a modification of the procedure described by Selye et al. (21). The other group was sham operated. The details of the surgical procedures were reported in our previous studies (22, 23). Briefly, the surgery was performed under chloral hydrate anaesthesia (36 mg/100g b wt, ip). The heart was exteriorised through the opening between the fourth and the fifth intercostal space. The left coronary artery was ligated with a prolene surgical thread (Ethicon 7.0) and the thorax was closed with surgical sutures (Ethicon 4.0). In the sham-operated rats the surgical procedure was similar, except that the coronary artery was not ligated. The rate of survival of the surgery was equal to 52%. After the surgery both groups of rats were given antibiotic (Taromentin, Polfa, 3 mg/100g b wt, ip) and placed back into their home cages.

**Tissue harvesting**

Four weeks after the surgery the rats were sacrificed by decapitation. The thorax and the abdomen were opened and the heart and the lungs were subjected to visual inspection. The heart and the left kidney from the rat having no pericardial or pulmonary adhesions were excised and placed on the glass platform, which was maintained at 0°C. The thorax was opened and the heart was removed. The cardiac ventricles were quickly separated from each other and from the atra. The size of the infarct was determined planimetrically, as described by Leenen et al. (18) with some minor modifications (22, 23). Briefly, the left ventricle was cut along the longitudinal axis, placed flat on the transparent graph paper and the circumferences of the left ventricle (including septum) and of the infarct zone on the internal and external walls of the left ventricle were outlined. The infarct and the total left ventricle surfaces were estimated as the number of square millimeters. In each individual case measurements performed on the external and internal surfaces were averaged and the ratio of the average infarct surface to the total average left ventricle surface was calculated. The size of the infarct in the individual rat was estimated as the percentage of the total left ventricle surface. In addition, the heart was subjected to histological examination in order to verify presence of the infarct scar according to the procedure described previously (22, 23). In 10 infarcted and 10 sham rats fragments of the left and right ventricles were harvested from the regions located at the distance of 1.5 – 2.0 mm from the infarcted region. Fragments of the cortex from the lower pole of the kidney and the internal medulla of the kidney were excised. All tissue fragments were immediately frozen in liquid nitrogen and stored at 80°C until mRNA and Western blot analyses were performed.

**RNA isolation and detection**

Semiquantitative PCR analysis of MR mRNA was performed according to the technique described by Bouaboula et al. (24) with modifications of Lipman et al. (25) and some adjustments elaborated in our Laboratory for MR mRNA determination (7). Fragments of the heart were homogenized in 4 - 5 ml TRIzol (Gibco - Invitrogen). Subsequently RNA was extracted according to Gibco - Invitrogen and its quantity was determined spectrophotometrically. Samples of RNA were diluted in DEPC treated water (Gibco - Invitrogen) to obtain concentration of 1µg/1µl. The residual genomic DNA was removed by digestion with DNAAse (Gibco - Invitrogen, 1U DNAAse/1µg RNA for 15 min at 37°C). The efficiency of DNAAse treatment was verified by checking whether PCR product can be detected in samples amplified without prior reverse transcription (RT). Transcription reaction was performed in 23 µl of reaction volume containing 0.5µg RNA, 0.5µg/µl oligo (dT)_{12-18} (Amersham), 0.1M DTT, 2.5x10^{-3} M dNTPs, 20 U of RNAase inhibitor, First Strand Buffer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV, Gibco - Invitrogen). At the beginning RNA and oligo (dT)_{12-18} were incubated for 15 min in 13 µl of reaction volume at 65°C. The remaining reagents
were added and 23 µl samples were incubated at 37°C for 60 min. Subsequently, the samples were heated at 95°C for 5 min and placed on ice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene. Total 1.0 µl of cDNA was used for GAPDH gene. The competitor DNAase quantity for GAPDH gene was estimated by means of spectrophotometric analysis and subjected to PCR reaction in a buffer containing 80 µM dNTPs, 0.3µM of specific antisense primer, 1U Taq DNA polymerase (Sigma) competitive standard and 1.0 µl of cDNA obtained in RT reaction. The final sample (1.0 µl) was amplified in PTC-100™ Programmable Thermal Controller (MJ Research). For MR gene expression 1.5 µl cDNA samples were used. The primers were purchased from Invitrogen Life Technology. The sequence of the primers and the size of the gene products are presented in Table 1. The standard was produced by PCR reaction in 30 cycles (94 °C, 1 min; 55 °C, 1 min; 72°C, 1 min). Amplification of MR mRNA was performed in thirty-five cycles (94 °C, 45 s; 60 °C, 30 s; 72 °C, 45 s). In case of GAPDH after 30 cycles (94 °C, 1 min; 55°C, 1 min; 72 °C, 1 min), 1 cycle (72 °C, 10 min) was performed. The specific PCR products were separated on 2.0 % agarose gel, containing 0.4 µg/ml of ethidium bromide and DNA size marker (123 bp ladder). Densitometric analysis of each band was performed by scanning the gel with Scion Image (ZeroDscan System, Scanalytic Inc). The results were expressed as Integrated Optical Density (IOD) and normalized for GAPDH IOD.

**Western blotting analysis**

Mineralocorticoid receptor protein expression was analysed by means of Western blotting. Fragments of the heart and kidney were homogenized in RIPA lysis buffer which was prepared with some modifications elaborated in our Laboratory. The buffer contained: 10 mM Tris-HCl, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Trion X-100; 10% glycerol; 0.1% SDS; 1mM PMSF and peptidase inhibitors leupeptin and aprotinin 10 µg/ml. After homogenization the probes were centrifuged for 20 min at 11600 x g at 4°C, and subsequently for 20 min at 20600 g at 4°C in Eppendorf Centrifuge 5417R. The supernatant was collected and frozen at 20°C. Protein concentration in the supernatant was determined by the Bradford method Sigma using bovine serum albumin (BSA, Sigma) as a standard. Probes containing 10 mg/ml of total protein were separated on 8% SDS-polyacrylamid gels for 45 min at 200V at room temperature. Molecular weight markers (Full-Range Rainbow, Amersham) were used in each individual gel. The transfer buffer contained 25 mM Trizma Base (Sigma), 192 mM glycine (Sigma) and 20% methanol (POCH, Gliwice), pH = 8.3. After transfer the membranes were rinsed in 0.5% Tween 20 in TBS (TTBS) (Bio Rad) and soaked at 4 °C for a night in blocking TTBS solution containing 5% skim milk. Subsequently, the membranes were washed in TTBS and incubated for 1 h with rabbit polyclonal antibody against mineralocorticoid receptor (Santa Cruz Biotechnology) diluted 1:666 in TTBS containing 0.1% skim milk. Next step involved washing in TTBS and incubation for 1 h with goat anti-rabbit

**Table 1.** Sequence and product size for glycerylaldehyde phosphate dehydrogenase (GAPDH) and mineralocorticoids (MR) mRNA genes

<table>
<thead>
<tr>
<th></th>
<th>GAPDH</th>
<th>MR</th>
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<tr>
<td>Sense 5’ primer</td>
<td>5’ - cataccatcttccaggage</td>
<td>5’ - ccaaggtcaccagcttc</td>
</tr>
<tr>
<td>Antisense 5’ primer</td>
<td>5’ - ctaagcagttggtggtgc</td>
<td>5’ - tcaccagcgactattgtct</td>
</tr>
<tr>
<td>competitor 5’ primer</td>
<td>5’ cataccatcttccaggageagtc cactggcagtctc</td>
<td>gene product size 270 bp 240 bp</td>
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secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at 1:2500 dilution in TTBS containing 0.1% skim milk. Incubation was performed at room temperature. Specific bands were visualised with chemiluminescence, ECL Western Blotting Detecting Kit (Amersham) and exposed to film that was immediately developed. The film was scanned and saved to a computer. The intensity of the bands was evaluated by means of a computer analysis using an image analysis software program ZeroDScan, and expressed in arbitrary units (A.U.).

Statistical analysis

Shapiro-Wilk test revealed that the data follow a normal distribution. Therefore one-way analysis of variance (ANOVA) was used to determine significance of differences between the respective groups of data. Pairwise comparisons between the individual groups of data were performed with the post hoc Tukey test. The following groups of data were compared: LV sham vs LV infarct; RV sham vs RV infarct; LV infarct vs RV infarct; renal cortex sham vs renal cortex infarct; renal medulla sham vs renal medulla infarct; renal cortex sham vs renal medulla sham; renal cortex infarct vs renal medulla infarct. Significance of linear regression slopes and elevations, and of correlation coefficients was determined using multiple regression analysis. The statistical tests were performed by means of Statistica software. The results were considered significant if P was less than 0.05.

RESULTS

The average surface of the infarct was equal to 30.2 ± 1.9% (range 23 - 40%) of the LV surface. Significant differences in expression of MR mRNA and MR protein in the heart were found between the infarcted and the sham rats (Figs 1, 2). In the left ventricle wall IOD MR mRNA/IOD GAPDH mRNA ratio was equal to 1.61 ± 0.29 in the infarcted rats and to 0.83 ± 0.15 in the sham rats [F(1,17) = 6.23; P< 0.02]. In the right ventricle wall IOD MR mRNA/IOD GAPDH mRNA ratio in the infarcted rats amounted to 1.31 ± 0.18 as compared to 0.64 ± 0.12 in the sham-operated rats [F(1,18) = 9.98; P < 0.005] (Fig. 1). Western blotting analysis revealed that in the left ventricle the difference in expression of MR mRNA between the infarcted and the sham rats was associated with significant difference in expression of MR protein (Fig. 2). In the left ventricle wall expression of MR protein was equal to 0.45 ± 0.02 A.U. and in the sham rats to 0.34 ± 0.004 A.U. [F(1,20) = 22.34; P < 0.001]. In the right ventricle expression of MR protein was similar in the infarcted (0.36 ± 0.001 arbitrary units) and in the sham rats (0.38 ± 0.016 A.U.) and did not differ from that found in the left ventricle of the sham rats. In both ventricles the slopes of the regression lines between the surface of the infarct and the expression of MR mRNA or MR protein were not significant. Similarly there was no significant difference between elevations of the regression lines for these parameters between the left and the right ventricle. Neither was significant correlation found between the size of the infarct and the expression of MR mRNA in the left or in the right ventricle wall.
In the kidney expressions of MR mRNA and MR protein in the cortex and in the renal medulla were not significantly different in the infarcted and the sham-operated rats (Figs 1, 2).

DISCUSSION

The present investigation reveals significant differences in expression of MR mRNA and MR protein in the heart of the infarcted and the sham-operated rats. It is also shown that the post-infarct expression of MR mRNA is significantly greater both in the left and in the right cardiac ventricle while expression of MR protein is increased only in the left ventricle wall.

Fig. 1. Expression of mineralocorticoid receptors (MR) mRNA in the sham-operated (control) and the infarcted rats in the left and right cardiac ventricle, renal cortex and renal medulla. IOD - integrated optical density; GAPDH - glyceraldehyde-3-phosphate dehydrogenase. P – significant difference between the infarcted and the sham-operated rats.
Increasing number of studies provides evidence that mineralocorticoids belong to the constellation of compounds exerting potent direct and indirect effects on the cardiovascular system (26 - 31). It has been shown that aldosterone plays significant role in development of cardiac hypertrophy and structural and electrical remodelling of the heart (26 - 30). During the post-infarct cardiac failure the impact of mineralocorticoids on the heart is significantly enhanced (31 - 34). Systemic blockade of mineralocorticoid receptors was found to ameliorate hemodynamics and cardiac performance during the postinfarct cardiac failure, both in the experimental studies and in the clinical trials (33, 34). The present study strongly suggests that the increased stimulation of the heart by mineralocorticoids during the post-infarct state may be caused not only by the

*Fig. 2. Expression of mineralocorticoid receptors protein in the sham-operated (control) and the infarcted rats in the left and right cardiac ventricle, renal cortex and renal medulla. IOD - integrated optical density. P – significant difference between the infarcted and the sham-operated rats.*
increased synthesis of these steroids but also by an increased availability of mineralocorticoid receptors. Our results are in agreement with those obtained by Yoshida et al. (14) in patients with the congestive heart failure (14). However, there are several essential differences between the former (14) and the present study. In the former study the samples were harvested from the human subjects suffering from the cardiac failure of different origin. The duration of the disease and pharmacological treatment received by the patients were not specified. In the present investigation the cardiac failure was produced exclusively by ligation of the coronary artery. The samples were harvested 4 weeks after the infarction under standardised conditions, when the animals fully recovered from the surgical trauma and were not subjected to any pharmacological treatment. Thus, it is justified to conclude that in the present study the augmented expression of MR in the heart of the infarcted rats was a consequence of processes initiated by the myocardial infarction itself. In the present investigation the hemodynamic parameters of the heart were not determined in order to avoid the influence of any additional interventions on expression of MR. However, in our previous studies (23, 35, 36) the myocardial infarct of the same size as that produced in the present experiments was associated with significant elevation of the left ventricle diastolic pressure. The latter finding strongly suggests that the rats used in the present investigation have developed the post-infarct heart failure.

The mechanism responsible for the enhanced expression of MR mRNA in the heart is at present unknown. Among the putative causes may be an increased production of cytokines occurring in the heart during the post-infarct state (37, 38). It is interesting that in the present study significant post-infarct increase in expression of MR protein was found only in the wall of the left cardiac ventricle despite significant elevation of MR mRNA in both ventricles. This finding suggests that some local factors generated in the wall of the infarcted ventricle (cytokines, other inflammatory factors, disturbances of the ionic milieu, excessive distension of the surviving cardiomyocytes) may play an ultimate role in determining MR mRNA and protein synthesis during the postinfarct state. In support of multiple causes of the increased MR expression in the heart during the post-infarct state is a lack of significant correlation between the size of the infarct and the expression of MR mRNA and MR protein in the cardiac ventricles.

Increased expression of MR mRNA and MR protein in the heart was not associated with significant changes of these parameters in the kidney. The myocardial infarct-induced cardiac failure is associated with changes in renal functions (36) due to activation of the sympathetic and renin-angiotensin-aldosterone systems and enhanced release of vasopressin and other compounds (1, 2, 15, 16, 19). The lack of changes in expression of MR mRNA and protein in the kidney during the post-infarct period indicates that the above factors do not exert major influence on synthesis of renal mineralocorticoid receptors during the post-infarct state.
In summary, the present investigation reveals that the myocardial infarction results in significant increase in expression of MR mRNA and MR protein in the left cardiac ventricle and MR mRNA in the right cardiac ventricle.

Acknowledgments: The authors are very grateful to Mrs. Małgorzata Kowalczyk, Marzanna Tkaczyk and Mr Marcin Kumosa for their skillful technical assistance. The study was supported by the grant from the Medical University of Warsaw (1MAW2) and by the State Committee for Scientific Research in Poland (KBN, grant 4P05A 074).

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Received: May 11, 2007
Accepted: November 5, 2007

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