Angiotensin II Converting Enzyme Inhibition Improves Survival, Ventricular Remodeling and Myocardial Energetics in Experimental Aortic Regurgitation

Arsenault et al: Improved Survival and Myocardial Energetics by ACEI in AR

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Abstract

Background—Aortic valve regurgitation (AR) is a volume overload disease causing severe eccentric left ventricular hypertrophy and eventually heart failure. There is currently no approved drug to treat patients with AR. Many vasodilators including angiotensin converting enzyme inhibitors have been evaluated in clinical trials but while some results were promising, others were inconclusive. Overall, no drug has yet been able to improve clinical outcome in AR and the controversy remains. We have previously shown in an animal model that captopril reduced left ventricular hypertrophy and protected LV systolic function but we had not evaluated the clinical outcome. This protocol was designed to evaluate the effects of a long term captopril treatment on survival in the same animal model of severe aortic valve regurgitation.

Methods and Results—Forty Wistar rats with AR were treated or not with captopril (1g/l in drinking water) for a period of 7 months to evaluate survival, myocardial remodeling and function by echocardiography as well as myocardial metabolism by μPET scan. Survival was significantly improved in captopril-treated animals with a survival benefit visible as soon as after 4 months of treatment. Captopril reduced LV dilatation and LV hypertrophy. It also significantly improved the myocardial metabolic profile by restoring the level of fatty acids metabolic enzymes and utilization.

Conclusions—In a controlled animal model of pure severe aortic valve regurgitation, captopril treatment reduced LV remodeling and LV hypertrophy, improved myocardial metabolic profile and survival. These results support the need to re-evaluate the role of ACEI in humans with AR in a large, carefully designed prospective clinical trial.

Key Words: aortic regurgitation, captopril, hypertrophy, metabolism, left ventricle, volume overload
Aortic valve regurgitation remains a disease without any proven effective treatment\(^1\). Patients with severe AR will develop over time severe left ventricular dilatation, eccentric hypertrophy and eventually heart failure. The current treatment strategy is essentially to wait-and-see, with a careful serial clinical and echocardiographic follow-up to detect progressive LV dilatation, decrease of LV function or occurrence of symptoms that would mandate aortic valve replacement surgery\(^1\). Many clinical trials have evaluated the efficacy of various drugs with vasodilator properties in the treatment of severe AR\(^2,3\). Unfortunately their results have been inconclusive. Angiotensin-converting enzyme inhibitors (ACEI) were tested in AR but conflicting results were obtained. It has been suggested that trial design and/or patient selection may be at least in part responsible for the inconsistency of the results\(^4\). We have previously shown in a controlled animal model of pure severe aortic valve regurgitation that ACEI captopril reduced left ventricular hypertrophy and protected LV systolic function\(^5,6\). However, we could not provide a clear mechanism to explain those protective effects. We also had not evaluated the effects of the treatment on the long term clinical outcome of the animals treated with captopril. This study was therefore designed to evaluate if captopril treatment improved survival in rats with severe aortic valve regurgitation. We also evaluated the effects of captopril on LV remodeling, hypertrophy as well as on myocardial metabolism in search of a potential protective mechanism.

**Methods**

**Animals:** Sixty adult Wistar rats were purchased from Charles River and divided in 3 groups: normal sham-operated (sham; \(n=18\)), untreated with aortic regurgitation (AR; \(n=25\)), captopril-treated with aortic regurgitation (AR-Cpt; \(n=21\)) (1g/l in drinking water). Treatment with captopril was initiated two weeks after the AR induction. All animals were evaluated daily by experienced technicians for the detection of signs of respiratory distress or pain. Animals were...
weighed weekly. Animals with respiratory distress were quickly euthanized. The protocol was approved by the Université Laval’s Animal Protection Committee and followed the recommendations of the Canadian Council on Laboratory Animal Care.

**Aortic regurgitation:** Severe AR induced by retrograde puncture of the aortic valve leaflets under 1.5% inhaled isoflurane anesthesia as previously described. Sham animals had their right carotid artery cannulated under anesthesia without puncture of the aortic valve. Animals were clinically evaluated daily by experienced animal laboratory technicians for the presence of signs of heart failure (increased respiratory rate/distress and/or peripheral edema) and were weighed weekly. At the end of the protocol surviving animals were sacrificed, hearts were quickly dissected and all cardiac chambers were weighed. LVs were snap-frozen in liquid nitrogen and kept at -80°C for further analysis.

**Echocardiography:** A complete M-Mode, 2D and Doppler echocardiogram was performed on the animals under 1.5% inhaled isoflurane anesthesia using a 12 MHz probe with a HD11XE echograph (Philips Medical Imaging, Andover, MA) immediately before and during surgery, after 3 months and 6 months. The echocardiogram after 2 weeks was performed to quantify AR before starting the protocol to ensure all animals still met the entry criteria. Left ventricular dimensions, wall thickness, fractional shortening, diastolic function, cardiac output, myocardial performance index were evaluated as previously reported.

**Small animal PET protocol:** Imaging experiments and data analysis were performed essentially as described before. Images were acquired using either a LabPET™ avalanche photodiode-based small animal PET scanner having a 4 cm axial FOV, or a Triumph™ dual modality
PET/CT scanner having a 8 cm axial FOV (Gamma Medica, Northridge, CA) at the Sherbrooke Molecular Imaging Centre. Care was taken to consistently use the same scanner for a given animal. Briefly, under isoflurane anaesthesia (1-1.5% at 1L/min oxygen flow), a catheter was installed in the caudal vein for the administration of the radiotracer. The animal was then positioned with the heart in the center of the scanner FOV and a 45-min gated dynamic acquisition was started 60 s before $[^{18}\text{F}]-\text{fluorothioheptadecanoic acid}$ ($[^{18}\text{F}]-\text{FTHA}$) (30-40 MBq, in 0.3 ml plus 0.1 ml flush of 0.9 % NaCl, respectively) was injected via the caudal vein over 30s. The animal was returned in his cage after each scan. Image dynamic data analysis was performed as described previously. Myocardial non-esterified fatty acid (NEFA) uptake (Km) and myocardial NEFA fractional uptake (Ki) were determined by a Patlak graphical analysis of the $[^{18}\text{F}]-\text{FTHA}$ data.

**Left ventricular volumes and ejection fraction:** The analysis was performed by dividing the cardiac cycle into eight gates on the basis of the R-R intervals using the last 15 minutes of the $[^{18}\text{F}]-\text{FTHA}$ list-mode dynamic gated acquisition. The analysis yielded the left end systolic (ESV) and diastolic (EDV) volumes from which the stroke volume (SV) and ejection fraction (EF) were computed as SV=EDV-ESV and EF=100*SV/EDV. The Corridor4DM software from Segami Oasis (Columbia, MD) was used for reorientation and cardiac data analysis, as previously described.

**Enzymatic activity determinations:** Left ventricle samples were kept at -80°C until assayed for maximal ($V_{\text{max}}$) enzyme activities. Small pieces of LV (20-30mg) were homogenized in a glass-glass homogenizer with 9 or 39 volumes of ice-cold extracting medium pH7.4 (250mM sucrose,
10mM Tris-HCl, 1mM EGTA) depending on the enzyme activity assayed. Enzymatic activities for hydroxyacyl-Coenzyme A dehydrogenase (HADH), hexokinase (HK) and citrate synthase (CS) were determined as previously described\textsuperscript{16}. Enzymatic activities for carnitine palmitoyl transferase (CPT) and malonyl-CoA decarboxylase (MCD) enzymatic activity determination were also previously described elsewhere\textsuperscript{17}.

**Analysis of mRNA accumulation by quantitative RT-PCR**

The analysis of LV mRNA levels by quantitative RT-PCR has been described in details elsewhere\textsuperscript{18}.

**Immunoblotting**

Crude LV homogenates were separated by SDS-PAGE. Immunoblotting was performed as described elsewhere\textsuperscript{19}. Membranes were hybridized with the indicated primary antibodies. All primary antibodies against the phosphorylated or the total form of the different signaling proteins (Erk 1/2, p38, Jnk, AMP kinase and Akt) were used at a 1:1000 dilution and were purchased from Cell Signaling Technology (Beverly, MA).

**Statistical analysis**

Results are presented as mean ± SEM unless specified otherwise. Comparison of variance between groups was performed using Bartlett’s and Brown-Forsythe tests. Data were log-transformed when variances were different between groups in order to equalize them. Inter-group comparisons were done using one-way ANOVA and Tukey’s post-test or Student’s t-test. Survival was analyzed by standard Kaplan-Meier analysis with log-rank test. Statistical
significance was set at a $p<0.05$. Data and statistical analysis were performed using Graph Pad Prism version 6.02 for Windows, Graph Pad Software (San Diego CA).

**Results**

**Survival data and animal characteristics (Figure 1):**

Captopril was well tolerated by all animals. Figure 1 shows the survival curves of untreated (AR) or captopril-treated (AR-Cpt) animals over a period of 210 days. All sham-operated animals were alive at the end of the protocol (not shown). Ninety-five percent of animals treated with captopril were alive after 7 months compared to only 68% in the untreated group. No animals in either groups developed signs of overt heart failure defined as excessive weight gain, labored breathing, peripheral edema or decrease of fractional shortening below 30% at echo. Most deaths were unexpected based on previous-day examination and were un-witnessed since they occurred overnight.

**LV remodeling, function and hypertrophy (Table and Figure 2):**

After 7 months, surviving animals underwent a final echocardiogram and their hearts were harvested subsequently for tissue analysis. Several randomly chosen animals also underwent a $\mu$PET scan for more precise LV volume and function measurements (Figure 2) as well as myocardial metabolism evaluation (see next sections). AR resulted in severe left ventricular hypertrophy and dilatation as demonstrated by the increased total heart and LV weight as well as end-diastolic and end-systolic diameters (Table). Captopril significantly decreased LV hypertrophy, dilatation and systolic dysfunction. The echocardiographic findings were corroborated by the $\mu$PET measurements of left ventricular end-diastolic volume, end-systolic volume and ejection fraction (Figure 2).
Markers of hypertrophy and extracellular matrix remodeling (Figure 3).

As expected, ANP and BNP gene expression were elevated in AR animals. Captopril treatment reduced ANP mRNA levels in AR animals but not those of BNP (Figure 3A). The relative gene expression of both the alpha and beta forms of myosin heavy chains was modified in untreated AR animals in which the alpha/beta ratio was strongly reduced. Captopril treatment normalized the expression of both MHCs in AR rats (Figure 3B). The mRNA levels of collagen 1, 3 and fibronectin were also measured in the LV samples. Figure 3C clearly shows that AR significantly increased the expression of collagen 1, 3 and fibronectin. Captopril treatment significantly decreased this overexpression with a return to close to normal values for collagen 1 expression.

Myocardial energetic metabolism and markers (Figures 4 and 5A-D)

Free fatty acid uptake was evaluated by μPET quantification as shown in Figure 4. LV fatty acid uptake was reduced in AR animals compared to controls. These changes in fatty acid uptake were predominant in the LV lateral wall. Captopril treatment normalized this parameter in AR animals. These findings were corroborated by the evaluation of various LV energy metabolism markers measured directly in myocardial samples (Figure 5). The carnitine palmitoyl transferase activity (CPT) which mediates the entry of fatty acids into the mitochondria was reduced in the LV of AR animals captopril treatment normalized activity levels. Fatty acid oxidation (FAO) capacity was impaired in the LV of AR rats as illustrated by the decreased HADH enzymatic activity (Figure 5A). Captopril restored this parameter to normal levels. Malonyl-CoA is an inhibitor of CPT activity and thus of FAO. The malonyl-CoA decarboxylase (MCD) is responsible for the conversion of malonyl-CoA to acetyl-CoA. MCD activity was reduced in untreated AR animals and captopril treatment normalized activity levels. FAT/CD36 is the cell membrane transporter.
implicated in the entry of fatty acids inside the cell: messenger RNA levels for FAT/CD36 were reduced in AR LVs and captopril treatment helped maintain its normal expression (Figure 5B). PPARα gene expression, a transcription factor implicated in the control of gene expression of various genes implicated in FAO (Figure 5B) as well as for PGC1α, a PPARα co-activator were both reduced in AR and captopril normalized their levels.

Conversely the first step of glycolysis, catalyzed by hexokinase, was increased in AR rats but captopril returned this parameter to normal while mRNA levels of pyruvate dehydrogenase that is responsible for the conversion of pyruvate to acetyl-CoA were reduced in AR animals but not in the captopril group (Figure 5C). We measured citrate synthase (CS) activity and aconitase 2 gene expression at the mitochondrial level (Figure 5D). In both AR and captopril-treated AR rats, CS enzymatic activity was slightly reduced. Aconitase 2 mRNA levels were reduced by AR but captopril partially restored these levels to normal.

G-protein receptor kinase 5 expression (Figure 5E):

The AT1 receptor is one of the possible substrate for the G-protein receptor kinase 5 (GRK5)20. GRK5 expression has been shown to be up-regulated in heart diseases21. In Figure 5E is illustrated the GRK5 mRNA levels in the LV of AR rats treated or not with captopril. AR rats expressed higher levels of GRK5 compared to controls and captopril treatment was able to almost normalize these levels.

Pro-hypertrophic pathways and the activation of Akt (Figure 6):

Some classical pro-hypertrophic pathways were also evaluated. AR did not affect the expression of Erk1/2, p38 or Jnk and neither did captopril treatment. However, AR significantly increased the expression of Akt-phosphorylated Akt ratio whereas captopril significantly prevented this
overexpression. The expression of AMPK was not affected by AR but its expression tended to be decreased by the administration of captopril.

**Discussion**

The main and most important finding of this study is the first demonstration that ACEI captopril improves survival in rats with pure severe aortic valve regurgitation. Our findings also confirm our previous observations of the benefits of captopril in AR. The controversy regarding the efficacy of vasodilators including ACEI in the treatment of AR is far from resolved. Recently, a meta-analysis of all the randomized clinical trials that evaluated vasodilators in AR showed that the evidence points towards the efficacy of vasodilators although a the wide discrepancy exists between the trials’ design and results. ACEIs were found almost always favorable in term of protection against LV dilatation and loss of ejection fraction. The authors nevertheless conclude that a large prospective trial remains necessary to confirm their findings considering the paucity of data.

It is also noteworthy that clinical outcome including survival has never been properly evaluated in most of the clinical trials published to date mostly due to their lack of statistical power. The most recent clinical trial from Evangelista et al evaluating enalapril, failed again to show or even suggest benefits in the drug treated group. Although it seems logical to assume that clinical outcome will parallel the benefits on LV remodeling and ejection fraction, the proof remains to be obtained. Despite its pitfalls, our animal model has the advantage of being controlled and without any confounding factors. A 7 month follow-up may reflect the sub-acute period in humans, it does enclose a true chronic phase in rats as we have previously shown in a paper characterizing this animal model. Our results show a clear effect on LV dilatation, hypertrophy, systolic function and myocardial metabolism and more importantly on survival.
These results provide support for the need to reevaluate ACEI in humans with AR in a large prospective clinical trial.

We report that captopril has a significant impact on myocardial energy metabolism. To our knowledge, no other study has evaluated the metabolic response of the heart to severe volume overload caused by AR and the effects of a drug treatment on this parameter. Whether the improvement in myocardial metabolism is the cause or the effect of the reduction of LV dilatation, hypertrophy and improved systolic function remains unknown and will need to be studied in other protocols.

In our study, captopril reduced LV dilation and volume and, consequently, wall stress. The severity of AR remained the same since AR is the result of an induced mechanical defect. Since the aortic valve lesion remained similar between both the untreated and the captopril-treated group, the LV volume overload was not eliminated by the captopril treatment. Nevertheless captopril was associated with lower ANP mRNA levels and a normal profile of MHC isoforms gene expression. The same was true for the expression of several components of the extracellular matrix. Although interstitial fibrosis is not an early feature of this animal model of eccentric LV remodeling, significant accumulation of collagen is present in later stages (6 months and beyond). Our results suggest that the dilated hearts of captopril-treated AR animals tolerated the abnormal hemodynamic overload and maintained a near normal myocardial metabolic profile.

We previously showed that blood pressure in AR animals treated with captopril is only slightly reduced and had similar findings in this protocol. This suggests that the hemodynamic contribution to the benefits of ACE inhibition although present is not the main and only factor involved.

The previously reported shift towards glucose utilization by the myocardium in compensated hypertrophy was not observed in our captopril-treated AR rats. We have recently reported this
shift in substrate uptake in 8-week AR animals. We observed that myocardial glucose uptake was increased while fatty acid was reduced in those animals. The overall myocardial oxidative capacity remained unchanged. In the present study, our data suggest similar findings after 7 months. Survivor AR rats did not show clinical signs of heart failure at the end of this study. Lungs weights were similar between groups arguing against the presence of sub-clinical pulmonary edema. An energy profile reminiscent of HF was not present in any of the AR animals, suggesting that the LVH state was still compensated. On a clinical standpoint, it would be advantageous to lessen LV dilation, ECM remodeling and maintain a normal energy substrate utilization for as long as possible.

Blocking angiotensin II (AngII) formation using ACE inhibition has shown its benefits in cardiac hypertrophy for a long time. AngII plays a major role in the development of hypertrophy and fibrosis. AngII increases norepinephrine release, the rate and force of cardiac contraction, and myocardial cell growth by its interaction with AT1-R. Its impact on myocardial metabolism is not well understood. Depending on the model studied, AngII is associated either with increased or decreased glucose utilization. Short-term AngII administration to cultured rat neonatal cardiac myocytes leads to increased glucose uptake while fatty acid uptake remains unchanged. Cardiac targeted overexpression of angiotensinogen has been shown to decrease FAO in HF mice but not in those displaying a compensated form of heart hypertrophy. A long-term treatment with AngII reduced FAO in cultured rat neonatal cardiomyocytes. In our model, we cannot conclude whether the observed effects of ACE inhibition on energy metabolism were the cause or an effect of the reduction of hypertrophy. Considering that ACE inhibition did not completely prevent hypertrophy and that the LV still had to cope with volume overload, the energetic demand of the myocardium was still probably increased.
We observed that Akt activation in the AR LV was reversed by ACE inhibition. The Akt signaling pathway is an interesting target since it is both pro-hypertrophic and stimulator of glucose utilization by the cell. Since the Akt cascade can be activated by various stimuli including integrins (mechanical cellular stretch) and G-protein-coupled receptors (GPCR) such as the angiotensin II receptor type I (AT1R)\textsuperscript{30, 31}, it is possible that effects of captopril in limiting the hypertrophic response may also have contributed to a reduced utilization of glucose. Moreover, Akt activation is linked to the down-regulation of PPARα and PGC1α, both stimulator of FAO\textsuperscript{32}. We have recently shown in the same animal model that LV remodeling can be influenced by diet manipulation and by drugs such as metformin and fenofibrate which have significant metabolic effects while lacking any hemodynamic effects\textsuperscript{17, 25}. This suggests that manipulating myocardial metabolism without hemodynamic effects can impact LV remodeling in AR.

GRK5 expression was increased in the LV of AR rats as previously observed\textsuperscript{19}. G-protein coupled receptor (GPCR) kinases are involved in their desensitization. GPCR-independent actions of GRK5 have recently been reported. GRK5 accumulates in the nucleus of myocytes after a hypertrophic stimulus. It enhances Gq-mediated cellular growth via its capacity to phosphorylate the histone deacetylase 5 kinase leading to MEF2 repression, a transcription factor regulating cardiac myocyte growth\textsuperscript{33}. GRK5 may therefore be another molecular target to study in our animal model of AR.

In summary, we report for the first time that a long-term treatment with captopril decreases sudden death in a rat model of chronic AR. These benefits on survival were accompanied in surviving animals with decreased LV hypertrophy and improved myocardial energetics. The mechanisms involved are obviously complex and further studies will be necessary to better understand the response of the myocardium to volume overload in the context of ACE inhibition.
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Disclosures

None.

References


**Table.** Animal characteristics and echocardiographic data in surviving animals

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Sham: sham controls; AR: AR controls; AR-Cpt: AR treated with captopril. LV: left ventricle, ind: indexed, EDD: end-diastolic diameter, ESD: end-systolic diameter, SW: septal wall thickness, FS: fractional shortening, HR: heart rate and bpm: beats per minute. Na: not applicable. Values are expressed as mean ± SEM. Number of animals per group indicated in parenthesis. ***: p<0.001 vs. sham animals and §§: p<0.001 and §§§: p<0.001 vs. untreated AR animals. Echo measurements obtained under inhaled 1.5% isoflurane anesthesia in surviving animals.
Figure Legends

Figure 1. Survival Kaplan-Meier curves of rats with severe chronic aortic valve regurgitation. Untreated (AR: black circles) or treated with captopril (AR-Cpt: white circles) over a period of 7 months.Inset table: number of animals alive at the indicated time after AR induction.

Figure 2. Left ventricular volumes and ejection fraction as evaluated by \[^{18}\text{F}]\text{-FTHA \(\mu\)PET. End diastolic (EDV) and end systolic (ESV) volumes were measured as described in the Material and methods section. The stroke volume (SV) is the difference between the EDV and ESV and the ejection fraction the ratio of SV on EDV. Results are reported as the mean of data obtained from four animals / group \(\pm\) SEM, ***: p<0.001 between sham and AR groups. §: p<0.05 and §§: p<0.01 vs. untreated AR group.

Figure 3. Evaluation by real-time quantitative RT-PCR of the LV mRNA levels of genes related to LV hypertrophy (A and B) and extracellular matrix remodeling (C). Results are reported in arbitrary units (AU) as mean \(\pm\) SEM (n=12/gr.). **: p<0.01 and ***: p<0.001 between sham and AR groups. §§: p<0.01 and §§§: p<0.001 vs. untreated AR group. Sham (sham-operated animals) group mRNA levels were normalized to 1. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; \(\beta\)MHC, myosin heavy chain alpha; MHC, myosin heavy chain beta; SERCA2a, sarcoplasmic reticulum Ca2+-ATPase; PLB, phospholamban; Col1: collagen I; Col3: collagen III; Fn: fibronectin.

Figure 4. Myocardial fatty acid (A) uptake in the LV myocardium of AR rats as evaluated by \(\mu\)PET compared to sham animals. Total (B) and regional (C) LV fatty acid uptake was estimated
in sham, AR and AR-Cpt animals. FTHA uptake was evaluated as described in the Materials and Methods section (n=4/gr.) and are expressed as the mean ± SEM. *: p<0.05 vs. sham and AR groups. §: p<0.05 vs. untreated AR group.

**Figure 5.** Enzymatic activity and gene expression of metabolic markers as well as gene expression of GRK5 in the LV of AR rats treated or not with captopril (Cpt). A) LV myocardial activity levels of enzymes implicated in fatty acid transport, ß-oxidation and control. Carnityl palmitoyl transferase (CPT), HADH (hydroxyacyl-Coenzyme A dehydrogenase) and malonyl-CoA decarboxylase (MCD) enzymatic activities were measured in LV homogenates from at least 10 animals in each group as described in the Materials and Methods. B) Evaluation by real-time quantitative RT-PCR of the LV mRNA levels of the fatty acid transporter (FAT/CD36), PPARα and PGC1α. Results are reported in arbitrary units as the mean ± SEM (n=12/gr.) Sham (sham-operated animals) group mRNA levels were normalized to 1. C) LV myocardial activity levels of the hexokinase (HK) and pyruvate dehydrogenase alpha 1 (PDHa1) gene expression levels. HK activity was measured in LV homogenates from at least 10 animals in each group. PDHa1 mRNA levels were evaluated by real-time quantitative RT-PCR from 12 animals per group. D) LV myocardial activity levels of the citrate synthase (CS) and acotinase 2 (Acot2) gene expression levels. CS activity was measured in LV homogenates from at least 10 animals in each group. Acot2 mRNA levels were evaluated by real-time quantitative RT-PCR from 12 animals per group. E) Evaluation by real-time quantitative RT-PCR of the LV mRNA levels encoding for GRK5 (G-protein receptor kinase 5). *: p<0.05, **: p<0.01 and ***: p<0.001 between sham and AR groups and §: p<0.05, §§: p<0.01 and §§§: p<0.001 vs. untreated AR animals.
Figure 6. MAP kinases, Akt and AMP kinase activation in AR rats treated or not with captopril (Cpt). Evaluation of the phosphorylated and the total protein contents for each kinase were performed by immunoblotting as described in the Materials and methods section. Results are reported in arbitrary units as mean ± SEM (n = 10–12/group) of the ratio of the phosphorylated content on the total protein content. Untreated sham (sham operated animals) group protein contents are fixed to one and are expressed in arbitrary units (AU). *: p<0.05 and ***: p<0.001 between sham and AR groups and §§: p<0.01 vs. untreated AR animals.
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