Study on the Effects of Losartan on Cardiomyocyte Apoptosis and Gene Expression After Ischemia and Reperfusion in vivo in Rats

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Summary: In order to study the effects of losartan on cardiomyocyte apoptosis following ischemia (0.5 h) and reperfusion (48 h) in vivo and bcl-2 and bax gene expression, TUNEL staining method, immunohistochemistry and in situ hybridization histochemistry (ISHH) were used to monitor the apoptotic cells, mRNA and protein of gene expression, respectively. Image processing system was used to quantitively dispose the positive metric substance of both immunohistochemistry and ISHH through the average optical density (OD) value. The number of the apoptotic cells were 38 ± 9 (control group), 0 ± 1 (sham operation group) and 9 ± 4 (losartan-treated group) in each visual field respectively with the difference among the groups being significant (P < 0.001). OD values of bcl-2 (ISHH) were 0.0742 ± 0.0203 (control group), 0.0564 ± 0.0093 (sham operation group) and 0.0764 ± 0.0145 (losartan-treated group) respectively, while OD values of bcl-2 (immunohistochemistry) were 0.1374 ± 0.0137 (control group), 0.0851 ± 0.0186 (sham operation group) and 0.1252 ± 0.0264 (losartan-treated group). bcl-2 gene expression was increased significantly in the control group and losartan-treated group as compared with sham operation group (P < 0.05). OD value of bax (immunohistochemistry) was 0.0972 ± 0.0230 (control group), 0.0518 ± 0.0143 (sham operation group) and 0.0621 ± 0.0142 (losartan-treated group). bax gene expression was decreased very significantly in losartan-treated group and sham operation group as compared with control group (P < 0.001). Bcl-2/bax ratio was 1.413 (control group), 1.376 (sham operation group) and 2.016 (losartan-treated group) respectively. The results indicated that losartan might inhibit cardiomyocyte apoptosis following ischemia and reperfusion. The mechanism might be that bax gene expression was inhibited to increase bcl-2/bax ratio.

Key words: losartan; ischemia/reperfusion; cardiomyocyte apoptosis; gene modulation

Along with the thrombolysis therapy, PTCA and the coronary artery bypass graft are used in the treatment of acute myocardial infarction (AMI), these method played the important roles for restoring the myocardial perfusion, rescuing agonal cardiomyocyte or reducing the extension of the myocardial infarction and protecting the cardiac function, but ischemia/reperfusion can cause the myocardial injury. Therefore the study of the myocardial ischemia and reperfusion injury become the clinical hotspot. It is affirmative that losartan has the therapeutic effects for AMI[1-2], but it is unclear for the mechanism of AMI therapy and reperfusion injury prevention and treatment. The purpose of this experiment was to study the effect of losartan on cardiomyocyte apoptosis and investigate the possible mechanism.

1 MATERIALS AND METHODS

1.1 Materials and Animal Grouping

Fifteen Wistar rats (cleaning class, provided by Zoology Department of Tongji Medical University) of both sexes, weighing 200—250 g were used. They were randomly divided into 3 groups: sham operation group, control group and losartan-treated group, with 5 rats in each group. Bax immunohistochemistry reagent kit (product serial No.: SA2030), bcl-2 immunohistochemistry reagent kit (product serial No.: SA2015b) and bcl-2 in situ hybridization histochemistry reagent kit (product serial No.: MK150) were provided by Wuhan Boster Biology Engineering Company. Cell apoptosis detecting reagent kits was provided by Germany Boehringer Mannheim Company. Losartan was the product of Moshadong Pharmacy Corporation (product No.: 98054).

1.2 Establishment of Animal Model

Following anesthesia with ether, thoracic cavity was opened along left breastbone third and fourth ribs. The heart was squeezed out from thoracic cavity and left anterior depression branch (LAD) was ligated at intersection between arterial cone and left cardiac ear. Heart was put back into thoracic cavity. Then the thoracic cavity was closed. After 30 min, heart was squeezed out from thoracic cavity with the same method. LAD was loosed and heart was put into thoracic cavity once again. The local incision was given penicillin to prevent infection and then skins sutured. The rats in sham operation group were subjected to same...
procedure but the LAD was not ligated. On the 48th postoperative h, the rats were decapitated and thoracic cavity was quickly opened to remove heart. Heart was washed with the cold normal saline, the ischemia/reperfusion area of the left ventricle wall was taken out and put into 4% polyformaldehyde fix solution containing 0.1% DEPC. Each cardiac muscle was divided into two formaldehyde fixation solution containing 0.1% DEPC. Each cardiac muscle was divided into two parts and was fixed for 6 h for hybridization histochimistry and 24 h for immunohistochemistry and cell apoptosis detecting respectively. Then they were put into cane sugar overnight and sliced into section of 25 μm thick by using AO incubator slicing machine (USA). The sections were mounted on slides that were disposed by 1:10 polylysine. Each cardiac muscle was divided into two parts and was fixed for 6 h for hybridization histochimistry and 24 h for immunohistochemistry and cell apoptosis detecting respectively. Then they were put into cane sugar overnight and sliced into section of 25 μm thick by using AO incubator slicing machine (USA). The sections were mounted on slides that were disposed by 1:10 polylysine solution, dried, refixed in fixing fluid for 5 min, washed by normal saline and stored at refrigerator for later use. The samples for cell apoptosis detection were prepared as follows: the sample to be fixed for 24 h was made into frost slices which were refixed in fixing fluid for 15 min, and dehydrated through gradient alcohol (concentrations were 60%, 70%, 80%, 90%, 100% respectively, 1 min in each procedure), dried in air and stored at a -20°C refrigerator for later use.

1.3 Administration
20 mg/kg losartan was given by stomach once a day. The first administration was 5th h before operation. Subsequently, the second and third administrations were given in an interval of 24 h. The rats in the control group and sham operation group was given the normal saline with the same cubage.

1.4 Immunohistochemical Detection
(1) Frost slices were washed by distilled water following the treatment of 0.3% H₂O₂/methanol solution for 30 min in room temperature. (2) Goat serum blocking solution was added into the sections for 10 min in room temperature. (3) Mice antibody against bcl-2 (or Bax) genic antigen was added into the sections, and 1 h later the sections were washed with 0.5 mol/L PBS solution at 25°C. (4) Biotinylized goat antibody (IgG) against mice was added into the sections, and 1 h later the sections were washed with 0.5 mol/L PBS solution at 25°C. (5) SABC was added into the sections, and 20 min later the sections were washed with 0.5 mol/L PBS solution at 25°C. (6) DAB showed colour. (7) After hematoxylin counter staining, dehydration, transparency and obstructing glass piece were performed.

1.5 ISHH Detection
(1) The frost slice was washed with 0.3% H₂O₂/methanol solution for 30 min in room temperature and then washed with distilled water. (2) Uncovering mRNA nucleic acid segment with protein enzyme K for 10 min at 37°C. (3) The digoxin-labeled probe was added onto the slides for hybridization. (4) The slides was washed with 2× SSC solution for 5 min × 2 times in 25°C distilled water and then with 0.1× SSC solution for 10 min at 37°C. (5) Adding blocking solution in room temperature for 20 min. (6) Adding mice antibody against digoxin for 30 min at 25°C and then washing with 0.5 mol/L TBS solution. (7) Adding biotinylized goat antibody (IgG) against mice for 20 min at 25°C, then washing with 0.5 mol/L PBS solution. (8) Adding SABC for 20 min at 25°C and then washing with 0.5 mol/L PBS solution. (9) DAB showed colour. (10) After hematoxylin counter staining, dehydration, transparency and obstructing glass piece were performed.

1.6 Procedures of TUNEL Staining Means for Detection of Cell Apoptosis
(1) The frost slice was washed with 0.01 mol/L PBS solution. (2) Adding 0.2 mol/L HCL solution for 15 min. (3) Washing with 0.01 mol/L PBS solution. (4) Adding 5 μg/ml protein enzyme K solution for 15 min. (5) Washing with 0.01 mol/L PBS solution. (6) Washing with 0.1% citric acid buffer. (7) Washing with 0.01 mol/L PBS solution. (8) Adding TUNEL solution for 60 min at 37°C. (9) Washing with 0.01 mol/L PBS solution. (10) Adding Converter-AP solution for 30 min at 37°C. (11) Washing with 0.01 mol/L PBS solution. (12) (NBT/BCIP) (X-phosphate/BCIP) showed colour for 5 to 20 min followed by dehydration, transparency and obstructing glass piece.

1.7 Quantitative Analysis
Apoptotic cells were observed under a microscope (×400) in each field of vision (100 fields of vision in each group) and then the average values were calculated as the representatives. The optic density (OD) values of immunohistochemistry and ISHH were measured by using image analysis system under a microscope×200. Ten fields of vision in each group were selected to measure respectively, then the mean values were calculated as the representatives.

1.8 Statistics Analysis
All statistic data were processed by using t-test of two sample mean difference.

2 RESULTS
2.1 Effect of Losartan on Rat Cardiomyocyte Apoptosis
The result was shown in fig. 1. There were significant difference among the 3 groups (P<0.001). Cardiomyocyte apoptosis number in losartan-treated group was obviously decreased as compared with that in control group, suggesting that losartan could prevent and cure cardiomyocyte apoptosis following ischemia/reperfusion.

2.2 Effect of Losartan on Rat Bax and Bcl-2 Expression and Bcl-2/Bax Ratio
The expression of rat bcl-2 in the control group and losartan-treated group was higher than in sham operation group (table 1, fig. 2 and 3 with the difference being significant (P<0.05).