Changes in the expression of c-fos & heat shock protein genes & blood flow velocity in the brain of rats undergoing myocardial ischaemia/reperfusion

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Background & objectives: Myocardial hypofunction could lead to the derangement of brain functions. The expression of c-fos and heat shock protein (hsp) genes was recognized as markers of neural cell injury. We undertook this study to investigate the influence of myocardial ischaemia and reperfusion (I/R) on these molecular events in the rat brain tissue and changes in mean arterial blood pressure (MAP) and the cerebral blood flow velocity (CBFV), to understand the basis of cerebral pathology following cardiac ischaemia and reperfusion.

Methods: Healthy rats (n=42) were randomly allocated into seven groups: sham-operated (A); myocardial ischaemia for 15 min, followed by 0.5, 1, 2, 4 and 6 h of reperfusion respectively (B2-B6) and hypovolemia at medium level (C). I/R rat models were established by ligating the anterior branch of the left coronary artery. Expressions of the c-fos, hsp70, hsp27 and hsp90 genes in the cerebrum, cerebellum, medulla oblongata and hippocampus, were studied with immunohistochemistry and in situ hybridization. The MAP and CBFV of the rats were also measured.

Results: The expressions of c-fos and HSP70 in brain tissue increased after myocardial ischaemia/reperfusion, with the strongest signal appearing in the hippocampus and cerebral cortex, while labeling for HSP27 and HSP90α were not detectable in any of the experimental groups. The expressions of c-fos mRNA and hsp70 mRNA shared the similar characteristics with their encoding proteins. MAP, reflection of cardiac function and cerebral blood flow decreased following cardiac ischaemia and reperfusion.

Interpretation & conclusion: Our findings suggested that the brain damages occurred from the early phase of myocardial I/R. The exact mechanism of cerebral tissue injuries induced by myocardial I/R is not known. Further studies need to be done to throw light on these aspects.

Key words Cerebral blood flow - c-fos - HSP - immunohistochemistry - myocardial ischaemia/reperfusion
Ischaemic heart disease (IHD) is a major cause of mortality in the western world. The reperfusion after ischaemia is not beneficial to the injured tissues, but is harmful. One of the conspicuous alterations after ischaemia/reperfusion (I/R) is the suppressed synthesis of general genes and the increased expression of some specific genes, such as the immediate early genes (IEGs) and the heat shock protein genes (hsps). Fos is the protein encoded by c-fos gene, a member of the IEGs family. It mediates long-term cellular responses to external signals, and may serve as a master switch to upregulate the expression of other genes. It is proved that Fos protein plays an important role in the cell growth, differentiation and in the recovery from reversible injuries. Heat shock proteins (HSPs), a series of products encoded by hsps gene family, are considered as molecular chaperones. They are crucial to the cytoprotection and the repair of cells and tissues. Alterations in the expression of HSPs are considered to indicate different pathological conditions, such as I/R damage, cardiac hypertrophy, and inflammation.

The expression pattern of the above genes in the myocardium after myocardial I/R had been investigated extensively, and the similar studies in the brain tissue after cerebral I/R were also performed. But, no report could be found on the changes of the above gene expression in the brain tissue induced by myocardial I/R so far, while, it was clinically observed that the myocardial hypofunction could lead to the deranged brain functions, and vice versa. Because the expressions of fos and HSPs in the brain tissue are recognized as markers of neural cell injuries, we investigated relevant gene expressions in the brain tissue of rats undergoing different periods of reperfusion after myocardial ischaemia. The changes in the mean arterial blood pressure (MAP) and the cerebral blood flow velocity (CBFV) in the I/R rat model were also recorded.

**Material & Methods**

The healthy Wistar rats were supplied by Animal center, Sun Yat-sen University of Medical Sciences, PR China. The primary mouse-anti-rat Fos, HSP70, HSP27, HSP90 monoclonal antibodies, biotinylated goat-anti-mouse secondary antibody and streptavidin-biotin-peroxidase complex (SABC) reagent kit were bought from Santa Cruz Corporation, California, USA. c-fos mRNA, hsp70 mRNA oligonucleotide probes and ISH kit were bought from Boster Biological Technology Company, Wuhan, PR China. The physiological polygraph system was bought from Nihon-Kohden, Tokyo, Japan. Transcranial Doppler (TCD) equipment was bought from DWL Company, Germany.

**Animal groups and the establishment of myocardial ischaemia/reperfusion rat model:** This study was carried out on 42 healthy Wistar rats weighing 250 ± 20 g, receiving a standard diet and water ad libitum. Rats were randomly allocated into seven groups with six animals each: group A, sham-operated (chest opened without ligation of vessels); group B2 to B6, myocardial ischaemia 15 min, followed by 0.5, 1, 2, 4 and 6 h of reperfusion respectively; group C, hypovolaemia at medium level (3 ml blood was taken out).

Rats were anaesthetised by intraperitoneal administration of 2 per cent sodium pentobarbital (40 mg/kg). The trachea was cannulated, and assisted respiration was maintained by the use of a Harvard ventilator. Body temperature was maintained at 38±1°C. Subsequently, by left thoracotomy through the fourth intercostal space, the heart was exposed. A 0/3 silk suture on a tapered crochet needle was passed around the left main coronary artery close to its origin. The thread was passed through 2 mm diameter polyethylene duct to function as an occluder. Following the surgical procedure, the animals were allowed to stabilize for 30 min before the coronary artery ligation. After 15 min of acute myocardial ischaemia, the occluder was released, allowing the reperfusion of previously ischaemic myocardium. State of the animals was monitored by EKG all the time. The elevation of S-T segment (0.1 mv recorded in the standard surface electrocardiogram) served as the marker of myocardial ischaemia, and fall of ST segment to nearly half the level of elevation marked the success of myocardial I/R.

**Specimen preparation:** Tissues from the heart were collected from all the groups in the two different parts:
(i) Central portion of ischaemic areas formed as a result of coronary artery ligation; and (ii) non-ischaemic areas from the right ventricle. Brain tissues from four areas, viz., cerebral cortex, cerebellum, hippocampus and medulla oblangata were collected from groups A, B2, B4, B5 and C. Some of the collected tissues were fixed in formalin, embedded in paraffin, and sections were collected on glass slides [cleaned with 95% ethanol, treated with (APES) solution] for hematoxylin (HE) and immunohistochemical staining. Fresh tissues stored at -70°C, were sectioned by a cryomicrotome for in situ hybridization.

**Immunohistochemistry:** Following antigen retrieval by microwaving for 30 min in 0.01 M citrate buffer (pH 6.0), the immunostaining was carried out by SABC method. The primary mouse-anti-rat Fos, HSP70, HSP27 and HSP90α monoclonal antibodies (1:100) were added onto the specimens, and incubated at 4°C overnight. After washing in PBS, the slides were incubated sequentially with the biotinylated rabbit-anti-mouse secondary antibody (1:200) for 1 h at room temperature, followed by SABC reagent. The immunoreaction was visualized by the chromogen 3,3’-diaminobenzidine (DAB). Tissues processed identically omitting the primary antibody formed the negative control.

**In situ hybridization:** Frozen sections were fixed in 4 per cent paraformaldehyde (pH 7.4) for 30 min, followed by the treatment with 0.3 per cent H$_2$O$_2$ at room temperature to inactivate tissue RNases. After washing with double distilled water, the sections were treated with protease K (1:100 in dilution), incubated in 20 µl hybridization buffer containing digoxin-conjugated probe (0.5 µg/ml) for 24 h at 37°C. After blocking with bovine serum albumin (BSA), the mouse-anti-Dig antibodies were allowed to react with the specimens for 30 min, followed by biotin-conjugated goat-anti-mouse IgG for 20 min. The slides were washed with 0.05M PBS, and then SABC reagent was added. The peroxidase reaction was developed with DAB. The slides were counterstained with hematein for 8 min. Two cases of nasopharyngeal carcinoma known to contain c-fos mRNA and hsp70 mRNA were routinely used as positive controls; two slides treated without probe were used as negative controls.

**Detections of the arterial blood pressure (MAP) and the cerebral blood flow velocity (CBFV):** By femoral arterial cannulation, the MAP was continuously monitored by the pressure detecting head of Physiological Polygraph. According to the method of Tamura et al$^{14}$ to establish the middle cerebral artery occlusion model (MCAO), the middle cerebral artery was exposed, and the detecting head of DWL TCD was placed directly on the surface of MCA to continuously measure the CBFV through MCA.

**Statistical analysis:** Animals were randomly selected from five central and four lateral parts in each brain tissue section. The immunostained neurons in each area were counted at 400× magnification. SPSS 10.0 software was used for analysis and values expressed as mean ± SD. Differences in regional measurements among groups were compared by one-way ANOVA, while between two groups by Student-Newman-Keulia test. $P<0.05$ was considered to be statistically significant.

**Results**

**Study of the myocardial tissue after myocardial I/R**

Fos and HSP70-labeled cells were detectable respectively at 0.5 and 1 h after I/R in ischaemic areas, while at 1 and 2 h in non-ischaemic areas. The expression intensity increased with the time of reperfusion, but it was weaker in non-ischaemic areas than ischaemic areas all the time. Fos and HSP70 positive cells were mainly distributed in the endocardium at the early phase, while the labeling in the epicardium became stronger with the reperfusion. Labeling for HSP27 and HSP90α were not detectable in all the experimental groups. The expression patterns of c-fos mRNA and hsp70 mRNA in the myocardium were similar to their encoding proteins respectively.

**Study of the cerebral tissue after myocardial I/R**

**Immunohistochemistry of Fos, HSP70, HSP27 and HSP90:** In the control group, there were few neurons, glial cells and vascular endothelial cells with weak Fos and HSP70 staining. In the experimental groups, the immunolabeling was detectable from 0.5 h after
I/R, and the intensity increased with further reperfusion. Fos and HSP70 proteins shared the similar distributive characteristics. Labeling for both the proteins were mainly observed at layer II in the cerebral cortex, while nearly undetectable at layer I. In hippocampus the staining was located at CA1 and dentate gyrus, while in cerebellum it was mainly located within the Purkinjes cells. The positively stained cells were difusely distributed in medulla oblongata, with higher intensity labeling of neuronal nuclei. In the four areas of the brain studied, the immunolabeling in hippocampus and cerebral cortex was most intense, followed by the medulla oblongata and cerebellum.

Expression intensities of Fos and HSP70 in all the brain areas were very low in the control rats (A) and the rats undergoing hypovolaemia at medium level (C). Positively stained cells were detectable from 0.5 h after I/R (B2). The differences in intensity of staining and numerical density of cells labeled among groups A, C and B2 were statistically significant. The number of positive cells continuously increased with the time of reperfusion, and the difference between groups B4 and B5 was more evident, compared with groups A and C respectively ($P<0.01$) (Figs 1 and 2).

**Fig. 1.** Number of Fos-positive staining cells detected in the different parts of brain tissues in the five groups of rats. A, sham-operated group; C, medium level hypovolaemia group; B2, B4 and B5, myocardial I/R groups (ischaemia 15 min, followed by 0.5, 2, 4 h of reperfusion respectively); Compared with group A: *$P<0.05$, **$P<0.01$; compared with group C: $> P<0.05$, $P<0.01$.

**Fig. 2.** Number of HSP70-positive staining cells detected in different parts of brain tissues in the five groups of rats. A, sham-operated group; C, medium level hypovolaemia group; B2, B4 and B5, myocardial I/R groups (ischaemia 15 min, followed by 0.5, 2, 4 h of reperfusion respectively); compared with group A: *$P<0.05$, **$P<0.01$; compared with group C: $P<0.05$.

**Fig. 3.** Number of HSP27-positive staining cells detected in different parts of brain tissues in the five groups of rats. A, sham-operated group; C, medium level hypovolaemia group; B2, B4 and B5, myocardial I/R groups (ischaemia 15 min, followed by 0.5, 2, 4 h of reperfusion respectively); compared with group A: *$P<0.05$, **$P<0.01$; compared with group C: $P<0.05$.

**Fig. 4.** Number of HSP90 $\alpha$-positive cells detected in different parts of brain tissues in the five groups of rats. A, sham-operated group; C, medium level hypovolaemia group; B2, B4 and B5, myocardial I/R groups (ischaemia 15 min, followed by 0.5, 2, 4 h of reperfusion respectively); compared with group A: *$P<0.05$; compared with group C: $P<0.05$. 
Only a few HSP27 and HSP90α positive cells were detectable in all the brain areas for all the groups (Figs 3 and 4). The differences among groups were not significant, except that between group B5 and group A or group C (P<0.05).

Compared to sham-operated (group A), the expression intensities of Fos, HSP70, HSP27 and HSP90α in the hypovolaemia group at medium level (group C) showed a tendency to increase (P>0.05), but they were still weaker than that of the I/R groups.

Expressions of c-fos mRNA and hsp70 mRNA in the brain tissue - c-fos mRNA and hsp70 mRNA were almost undetectable in the brain tissues of the sham-operated rats, while they were detectable at 0.5 h after I/R. The strongest expression intensity appeared in the hippocampus, followed by cerebral cortex, medulla oblongata and cerebellum. The enhancement of the expression was time dependent, with the highest level reaching at 2 h after I/R (Fig. 5). The distribution characteristics of hsp70 mRNA and c-fos mRNA in the brain tissues were similar to their encoding proteins detected by immunohistochemistry. The expression intensities of c-fos mRNA and hsp70 mRNA in hypovolaemia group were stronger than that of the control group (P<0.01), but still weaker than that of the I/R groups (P<0.01).

Influence of myocardial I/R on MAP and CBFV in rats

The MAP values recorded from different I/R groups were 82.8 ± 7.19, 95.1±11.7 and 97.5 ± 10.8 mmHg 0.5, 2 and 4 h after I/R, respectively. The values were significantly lower (P<0.01) than that of the control group (131.7 ± 12.75 mmHg). The reduction of this index in medium level hypovolaemia group (77.6 ± 8.57 mmHg) was similar to that of the I/R groups, and significantly lower than the control group (P<0.01).

The mean CBFV value was 29.9 ± 5.42 cm/s in the control group. It decreased significantly after I/R, at 0.5 h after I/R (P < 0.01). The mean CBFV values in the different experimental groups were:

![Fig. 5. Expressions of hsp70 mRNA in various brain regions at 2 h of reperfusion after myocardial ischaemia, detected by in situ hybridization, with 3-3'-diaminobenzidine (DAB) and hematoxylin counterstaining. Clear and the strongest hybridization signals were shown in the cells of the hippocampus (C) and cerebral cortex (A), while the signals were weaker in the cerebellum (B). The positive cells were marked by arrows. A. positive cells in the cerebral tissue, x200; B. positive cells in the cerebellar tissue, x200; C. positive cells in the hippocampal tissue, x100.]
19.6 ± 2.76, 22.7 ± 3.06, 25.5 ± 3.17 cm/s at 0.5, 2 and 4 h after I/R, respectively. The CBFV value of the medium level hypovolaemia group (25.8 ± 2.78 cm/s) was slightly lower than that of the control group (P<0.05), but it was obviously higher than that of the 0.5 h I/R group (P<0.01).

Discussion

Myocardial ischaemic injury could lead to the expression of IEGs and hsp genes in the cardiac tissue, and cerebral ischaemia could also induce the altered gene expressions in rat brain tissues. However, there are no reports related to the influence of cardiac ischaemia/reperfusion on the expression of these genes and associated proteins in the brain. It is known that heart diseases can lead to changes in brain structures and cause altered brain functions, including the alteration of neurotransmitter turnover. So, it was important to evaluate the expression characteristics of c-fos and hsp genes in the brain tissue during myocardial I/R.

The encoding proteins of hsp genes, HSPs are classified into five families on the basis of molecular mass. The most important member, HSP70, is induced in all the cell types by a wide variety of injurious stimuli, and plays an important role in the myocardial protection against stress-induced damages, including ischaemia. The higher level of HSP70 in the serum was found to be associated with the lower risk of cardiac injury, while its reduction was significantly correlated with the high incidence of postoperative atrial fibrillation after the cardiac surgery. But recently, Olsson et al showed lack of neuroprotection by hsp70 overexpression in mouse model. The result of Fos and HSPs labeling in myocardium during the course of myocardial I/R in this study was in accordance with the previous studies. Our results indicated that fos and hsp70 mRNA and their encoding proteins were expressed in different anatomical areas of rat brain after myocardial I/R. The highest level of expression intensity appeared in the parts of brain that were prone to ischaemia (hippocampus and cerebral cortex). Low level of ischaemia caused by induced hypovolaemia resulted in weaker expression of stress related genes and the constitutive proteins unlike myocardial I/R groups. Since the expressions of Fos and HSPs were often considered as the indicators of the tissue injury, the results indicated that the stress reaction in the brain tissue was caused by myocardial I/R from the early phase. Similarly, the mean arterial pressure was also reduced significantly following induced hypovolaemia and ischaemia/reperfusion in the heart. The CBFV was less affected by induced hypovolaemia, in comparison to I/R group, especially in 0.5 h I/R group. With progression of time, the CBFV appeared to recover. It suggested there might be other factors, besides mean arterial pressure contributing to alteration in CBFV in the myocardial I/R groups. Some researchers considered that the cerebral vessel autoregulation mechanism is abnormal during the myocardial ischaemia. Our results suggested that although the decrease of MAP after myocardial I/R was not beyond its autoregulatory range (>60 mmHg), histologically discernable cerebral lesions had occurred, while induced simple hypovolaemia did not cause apparent lesions. The difference in CBFV especially during early periods might also explain why the expressions of Fos and HSPs in the brain induced by myocardial I/R changed more evidently than the simplex hypovolaemia.

The exact mechanism of cerebral tissue injuries induced by the myocardial I/R is not yet clear. It is probable that once the myocardial I/R and the accompanied arrhythmia has occurred, the fall in blood pressure activates sympathetic nervous system leading to cerebral vasospasm, fall in CBFV and resultant compensatory molecular and proteomic alterations. Further studies on similar line will provide insight into mechanism of cerebral injury following ischaemic cardiac lesions, which have wider clinical implications.

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References


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