Original Article

Effect of dyslipidemia on intima-media thickness of intra- and extracranial atherosclerosis by regulating the expression of hsp70 in rabbits

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Abstract: The aim of this study was to explore the effect of dyslipidemia on intima-media thickness (IMT) of Intra- and extracranial atherosclerosis by regulating the expression of heat shock protein 70 (HSP70) in rabbits. Twenty-seven male white rabbits were randomly divided into normal control group A, high fat group B and high fat + endothelial injury operation group C (each group was 9), we measured lipids and obtained tissues from different cerebral arteries including Bilateral common carotid artery (CCA), Internal carotid artery (ICA), middle cerebral artery (MCA) and vertebral artery (VA). Pathological analysis were done, western blot analysis was used to detect the expression of HSP70 in CCA and MCA. The Serum lipid levels were overall significantly increased at 12th week in Group B and Group C compared to normal control (P < 0.05); at 12th week, the IMT of CCA and MCA in group B and C were showed significant increment compared with Group A; the correlation between HDL/CHOL/LDL and IMT of different cerebral arteries are as follows: MCA > ICA > CCA > VA; between TG and IMT of different cerebral arteries: VA > ICA > MCA > CCA; the expression of HSP70 from MCA were increased compared with CCA in group B and group C (P < 0.05). Significant positive correlations were observed between hyperlipidemia and different cerebral arteries. Hyperlipidemia has more impact on IMT of intracranial cerebral arteries. The expression of HSP70 from intracranial cerebral arteries is significantly increased. The mechanisms underlied was speculated that might be involved in inhibiting the inflammatory via HSP70.

Keywords: High fat, HSP70, dyslipidemia, cerebral atherosclerosis, intima

Introduction

Atherosclerosis is a systemic vascular disease characterized by complicated pathogenesis involving endothelial dysfunction, Lipid infiltration, inflammation, oxidative stress and so on. Cerebral atherosclerosis is a major cause of ischemic stroke [1, 2]. Kim et al [3] showed that the prevalence of risk factors and stroke mechanisms were different between Intracranial atherosclerosis (ICAS) and extracranial atherosclerosis (ECAS), and found that hyperlipidemia was more closely associated with ECAS, it was consistent with a cross-sectional study conducted in New York [4], but Wong et al [5] showed that hyperlipidemia was more related to ICAS, hyperlipidemia which is widely accepted as the risk of stroke, has been insufficiently studied in cerebral ischemia patients with different cerebral atherosclerosis. Although hyperlipidemia is widely accepted as a major risk factor for coronary artery and peripheral vascular diseases, its role in ICAS and ECAS is still controversial [4-7].

Previous studies showed that ischemia can initiate the expression of the heat shock proteins (HSP) to maintain the integrity of cells, and several studies have reported that high expression of HSP70 could reduce overall lesion size and limit the tissue damage within the lesion of permanent cerebral ischemia [8]. It was positively related to the risk of vascular disease [9-11], HSP have been implicated in atherogenesis, and increased circulating level of HSP70 might play a role in initiation and/or progression of atherosclerosis through perturbation of CD4+ CD28null cells [12]. In previous studies, we
have found that the severity of ischemic stroke was correlated with the HSP70 expression level, the interactions between hypertension and HSP70 can increase the risk of cerebral ischemia [8], besides, Lin et al [13] have demonstrated that in the hyperlipidemic rabbits model, heat shock proteins (HSP) enhanced fatty streak and macrophage infiltration in atherosclerotic lesions, which may be mediated by elevated low-density lipoprotein receptor expression. Thus, the expression level and function of HSP70 may be closely related with the outcome of cerebral infarction.

Why does the impact of hyperlipidemia on ICAS and ECAS have widespread controversy? And which is intracranial and extracranial atherosclerosis more closely associated with the expression of HSP70? These questions always troubled us at the beginning of the subject. There has no related articles investigated the effect of dyslipidemia on IMT of Intra- and extracranial atherosclerosis by regulating the expression of HSP70. In order to figure out the interactions between hyperlipidemia and the expression of HSP70 in ICAS and ECAS. Thus, the principal aim of our study was to investigate the effect of dyslipidemia on IMT of Intra- and extracranial atherosclerosis by regulating the expression of HSP70. We created a cerebral atherosclerosis model of rabbits with high fat and endothelial injury operation, and further discussed about the impact of elevated blood lipids and hyperlipidemia on ICAS and ECAS.

Materials and methods

Animals and diets

Male New Zealand White rabbits (n = 27, weight 2.0 to 2.5 kg; provided by Animal Laboratory of Tongji University) were used in this study. They were fed regularly with chow for one week, and randomly divided into three groups: 9 were control group (A), 9 were high fat group (B), and 9 were high fat + endothelial injury operation group (C). Animals in all these groups, except the controlled group, were fed with high fat diet (2% cholesterol, 6% peanut oil and 92% basic feedstuff) for twelve weeks. All rabbits were fed with restricted diets with equal numbers of calories and provided with free access to water. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Animal models

We used the improved air-drying endothelial injury operation method based on a previous study [14]. Experiments were approved by the Animal Experiments Committee of Tongji University. Anesthesia was maintained with 3% pentobarbital sodium at the amount of 1. ml per kg via auricle vein. A midline incision was given in the animal’s neck and left common carotid artery was isolated. Air-drying endothelial injury technique was induced by applying nitrogen flow through the carotid artery. The left carotid artery was exposed and ligated at two points 2.0-2.5 cm apart by using bulldog clamp. A children scalp needle was inserted into the proximal end of the segment, and punctured through the distal end of this segment. The lumen was rinsed with saline, and a stream of dry nitrogen was allowed to flow through the segment at 250 ml/min for 5 minutes. The lumen was rinsed with saline again and the needle was removed. Then, a wet gauze was used to press on the ligated segments for 3-5 minutes before removal of two bulldog clamps. Circulation was re-established, hemostasis was ensured and the incision was closed. The right carotid artery was manipulated but not processed to air-drying endothelial injury to use it as control. Blood samples were taken from a marginal auricle vein after 12 h fasting and the level of plasma cholesterols (CHO), triglycerides (TG), low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) were measured at the end of the 4th, 8th, 12th week.

Histopathological examination

The rabbits of groups A, B and C were sacrificed in 4th, 8th and 12th week after surgery respectively. The artery segments (including common carotid artery, internal carotid artery, middle cerebral artery, vertebral artery, and basilar artery) were collected and stored at 4% paraformaldehyde for histomorphometry observation. Then, pathological analysis was done by Hematoxylin and Eosin (HE) stain. Images were observed under Optical microscope, and then intima-media thickness (IMT) measurement was done using Image Pro Plus 6.0.

Western blot

Protein lysates were separated on one-dimensional 10% SDS-PAGE gels, and then specimen proteins were transferred onto polyvinylidene
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Table 1. The blood lipids comparison between different groups and time (mmol/L, X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>TG</th>
<th>CHOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>0.56±0.25</td>
<td>0.40±0.48</td>
<td>0.92±0.26</td>
<td>1.15±0.74</td>
</tr>
<tr>
<td>8th</td>
<td>0.49±0.08</td>
<td>0.37±0.18</td>
<td>0.59±0.24</td>
<td>0.81±0.03</td>
</tr>
<tr>
<td>12th</td>
<td>0.48±0.03</td>
<td>0.45±0.02</td>
<td>1.54±0.10</td>
<td>1.27±0.01</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>6.65±0.91*</td>
<td>31.10±12.47*</td>
<td>0.79±0.36</td>
<td>33.48±8.07*</td>
</tr>
<tr>
<td>8th</td>
<td>9.00±3.00*</td>
<td>47.96±10.28*</td>
<td>1.69±1.37</td>
<td>40.26±2.96*</td>
</tr>
<tr>
<td>12th</td>
<td>10.93±2.13*</td>
<td>50.80±4.25*</td>
<td>4.22±2.88*</td>
<td>41.27±0.93*</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>8.96±3.04★</td>
<td>43.58±15.73★</td>
<td>1.43±0.92</td>
<td>36.64±7.39★</td>
</tr>
<tr>
<td>8th</td>
<td>9.65±1.46★</td>
<td>52.76±5.51★</td>
<td>3.26±1.01★</td>
<td>41.90±2.69★</td>
</tr>
<tr>
<td>12th</td>
<td>10.64±2.25★</td>
<td>57.61±1.72★</td>
<td>3.39±1.17★</td>
<td>43.63±2.44★</td>
</tr>
</tbody>
</table>

Values are expressed in terms of mean ± SD. *Statistical significance with P < 0.05 when compared between control and high fat group; ★ statistical significance with P < 0.05 when compared between control and high fat + endothelial injury group.

Figure 1. The average changes of different lipids between groups.

Difluoride membranes, the membranes were blotted with mouse-anti-Hsp70 antibody (1:100 dilution, abcam, cambridge, UK) and scanned. Densitometric analysis was performed using Quantity One software (Bio-Rad). Finally developed with the use of enhanced chemiluminescence reagents (ECL, Amersham, Del). The band intensity was determined by a gel image analysis system (Bio-Rad) and normalized with GAPDH.

Statistical analysis

SPSS for Windows (release 17. 0. 0 SPSS Inc, Chicago IL) was used for related data analysis.

Analysis of differences in experimental and control group was determined by one-way ANOVA, P < 0.05 was taken as statistically significant and P < 0.01 was taken as statistically highly significant. Spearman correlation analysis was used to analyze the correlation between different cerebral arteries and serum lipids.

Results

Serum lipid profile

From Table 1, blood lipid level was found to increase over the time in Group B (high fat), LDL and CHOL showed significant increment. Similarly, we can observe the changes of various lipid levels in different groups in Figure 1, LDL and CHOL were found to increase significantly in Group C (high fat + endothelial injury operation group).

Pathological section

In Figure 2, we could observe that the degree of AS lesion was gradually increased in endothelial injury group by comparing postoperative CCA in Group A, Group B and Group C at 12th week. In Figure 3, we found that the simple high fat feeding could lead to the formation of AS, high fat + endothelial injury could further aggravate the degree of AS lesion.

The contrast of different cerebral arteries segment

There already has reports proved that IMT measurements are a validated surrogate end point for atherosclerosis and vascular disease risk [15]. IMT measurements can provide data on the efficacy of novel lipid modifying in High fat diet. To estimate atherosclerosis progression between Group A, Group B and Group C, we used cross-sectional standardized IMT measurements in 3 groups, Because measurements were standardized, we could extrapolate atherosclerosis progression estimates from these cross-sectional data for each of the groups. The IMT of different cerebral arteries were measured in all subjects and combined to per-subject averages. From Table 2, we found that IMT increase with age was at least twice as
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Large in 12th of Group C as in CCA and MCA. High fat can cause IMT thickness in different cerebral arteries.

**Comparative analysis between blood lipids and IMT thickness**

Table 3 showed the spearman correlation analysis between blood lipids and IMT thickness in different cerebral arteries segment. At 12th week, the correlation between HDL and IMT of different cerebral arteries segment is as follows: MCA (spearman correlation coefficient = 0.792) > ICA > CCA > VA; between CHOL and IMT of different cerebral arteries segment: MCA (spearman correlation coefficient = 0.841) > ICA > CCA > VA; between TG and IMT of different cerebral arteries segment: VA (spearman correlation coefficient = 0.669) > ICA > MCA > CCA; and between LDL and IMT of different cerebral arteries segment: MCA (spearman correlation coefficient = 0.876) > ICA > CCA > VA.

**Western blot analysis**

To determine whether the expression of HSP70 was different between intracranial and extracranial cerebral atherosclerosis, we observed the expression of HSP70 in CCA (which is represented as extracranial cerebral artery) and MCA (which is represented as intracranial cerebral artery) at 12th week. As shown in Figure 4A, with increased of blood lipid over the time, there was higher expression in Group C both CCA and MCA, besides, there was higher
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Table 3. The spearman correlation analysis between blood lipids and IMT thickness in different cerebral arteries

<table>
<thead>
<tr>
<th>Blood lipids</th>
<th>Analysis objects</th>
<th>Spearman correlation Coefficient between lipids and cerebral arteries</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-density lipoprotein (HDL)</td>
<td>IMT of CCA</td>
<td>0.721</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of ICA</td>
<td>0.545</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of MCA</td>
<td>0.792</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of VA</td>
<td>0.496</td>
<td>0.051</td>
</tr>
<tr>
<td>Cholesterol (CHOL)</td>
<td>IMT of CCA</td>
<td>0.630</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of ICA</td>
<td>0.576</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of MCA</td>
<td>0.841</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of VA</td>
<td>0.590</td>
<td>0.016*</td>
</tr>
<tr>
<td>Triglyceride (TG)</td>
<td>IMT of CCA</td>
<td>0.456</td>
<td>0.011*</td>
</tr>
<tr>
<td></td>
<td>IMT of ICA</td>
<td>0.502</td>
<td>0.008*</td>
</tr>
<tr>
<td></td>
<td>IMT of MCA</td>
<td>0.574</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td>IMT of VA</td>
<td>0.669</td>
<td>0.003*</td>
</tr>
<tr>
<td>Low density lipoprotein (LDL)</td>
<td>IMT of CCA</td>
<td>0.720</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of ICA</td>
<td>0.609</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of MCA</td>
<td>0.876</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>IMT of VA</td>
<td>0.675</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

*Statistical significance at P < 0.05.

Figure 4. Western blot analysis of HSP70 levels. In (A), the gels show the immunoblots of HSP70 and GAPDH, HSP70 at 70 kDa, and GAPDH at 36 kDa. In (B), the bars show the relative protein levels of the immunoblots (mean ± SD from experiments). ▲ P < 0.05 when compared between Group A and Group B in MCA; ★ statistical significance with P < 0.05 when compared between Group A and Group B in CCA.

As hyperlipidemia is one of the most important risk factors for atherosclerosis that is associated with oxidative stress and endothelial dysfunction in cerebral arterioles [17], it could promote microvascular dysfunction before larger vessels with atherosclerosis. The altered microvascular function during hypercholesterolemia produces exaggerated tissue injury responses to ischemia and reperfusion and other stimuli (eg, endotoxemia) [18-20]. Although hyperlipidemia is widely accepted as a major risk factor for coronary artery and peripheral vascular diseases, very few researchers paid much attention to the influence of hyperlipidemia on ICAS and ECAS at present, but studies have showed that Asians and Africans were more likely to have ICAS, particularly middle cerebral artery, basilar artery and its branches. In contrast, Americans and Europeans had more frequent ECAS; particularly proximal segment of internal carotid artery had higher percentage of about 28% [21-24]. This variation might be related to various risk factors, such as cerebral artery anatomy, environmental habits, races, genetics, risk factors (blood pressure, metabolic syndrome, hyperlipidemia, etc) and its related gene expression. Our study focused on the effect of hyperlipidemia on IMT of Intra- and extracranial atherosclerosis. Which is mostly influenced by Intra- or extracranial cerebral atherosclerosis? Our results shown that the maximize correlation of HDL, CHOL, LDL and the IMT of different cerebral arteries are MCA (correlation coefficient respectively are 0.792, 0.841, 0.876), the maximize correlation of TG and the IMT of different cerebral arteries is VA (spearman correlation coefficient = 0.669). Our study proved that dyslipidemia is not only positive related to the differences of Intra- and extracranial cerebral atherosclerosis, but also aggravated the degree of IMT in intracranial cerebral atherosclerosis. Kim et al [3] considered that ICAS is more prevalent than ECAS, with an approximate ratio of 7:3, confirming the previous notion that ICAS is a more important cause of stroke than ECAS in Asian populations, besides, Wong et al [5] considered that the lipid level is higher in the intracranial artery group, it is similar to our results.

Discussion

Atherosclerotic lesions begin with an inflammatory reaction followed by smooth muscle proliferation and thickening of the arterial wall [16].
To clarify the discrepancy, we analyzed the possible related gene expression including precursor protein (albumin A chain), tropomyosin alpha 1 chain, HSP70, alpha smooth muscle actin, Beta galactose binding agglutinin, tropomyosin alpha-4 chain isoform 2, cell keratin 9, single octylic acid glyceride beta 2, and we found the expression of HSP70 in Intra- and extracranial cerebral atherosclerosis had significant differences through our experiment. The heat shock proteins (HSPs) are expressed by cells in response to stresses such as high temperature, sheer stress, and free radicals, including oxidized LDL cholesterol that cause complement-mediated endothelial injury and thus to accelerate atherogenesis [9, 16, 25]. HSPs have been shown to be important mediators of protective pathways as well as targets for autoimmunity leading to atherosclerosis [16, 26, 27]. Higher serum levels of HSP70 are associated with atherosclerotic intimal thickening [28]. More recently, Hsp70 has been shown to suppress inflammation and tissue damage via a mechanism that involves an enhanced regulatory response mediated by antigen-specific IL-10 production [29].

Degnan and Zheng et al [21, 30] suggest that overexpression of HSP70 can protect brain against ischemia via an anti-inflammatory mechanism. Previous studies showed that heat shock protein (HSP70) was positively related to Intra- and extracranial atherosclerosis, but which is intracranial and extracranial atherosclerosis more closely associated with the expression of HSP70 has not yet been elucidated. Our study pointed out that the protein expression of HSP70 in intracranial cerebral arteries (MCA) is significantly increased compared with in extracranial cerebral arteries (CCA) (P < 0.05), consistent with previous research showing that increased expression of Hsp70 lead to a neuroprotection effect. We speculate on that might be involved in the apparent atherosclerosis protective properties of this protein via anti-inflammatory mechanisms in ICAS compared with ECAS. More studies are necessary for better understanding participation of the HSP70 in the inflammatory response and especially its actions as anti-inflammatory agent in ICAS and ECAS.

A limitation of our study is its small sample of dyslipidemic rabbits. It would be important to confirm these findings in a larger sample and perhaps to examine in greater depth the relation in dyslipidemic patients with ICAS and ECAS. Furthermore, the relationship between the development process of atherosclerosis over time and hyperlipidemia still needs further studies. Although there still are controversies, hyperlipidemia has generally been considered a risk factor more closely related with ECAS than ICAS. Our results are not corroborated with generally prevailing idea on this issue; it may be associated with the differences between rabbit and human. It is still needs further studies.

In conclusion, the impact of hyperlipidemia to Intra- and extracranial cerebral atherosclerosis is different. Hyperlipidemia was found to be more related to IMT of intracranial cerebral arteries; besides, the expression of HSP70 in intracranial cerebral arteries is higher than that in extracranial cerebral arteries.

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Disclosure of conflict of interest

None.

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