Psychological stress increases expression of aortic plaque intercellular adhesion molecule-1 and serum inflammatory cytokines in atherosclerotic rabbit model

Muwei Li¹, Xianpei Wang¹, Lei Yang¹, Chuanyu Gao¹, Yexin Ma²

¹ Department of Cardiology, Henan Provincial People’s Hospital, Zhengzhou 450003, China
² Department of Cardiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430020, China

Background and objective  Plaque rupture, platelet aggregation, and thrombogenesis are the main mechanisms of acute coronary syndrome (ACS), and inflammation factors play key roles in plaque instability. Psychological stress promotes acute inflammatory response, leading to increased circulating levels of C-reactive protein (CRP), IL-6, and serum intercellular adhesion molecule (sICAM)-1. But it is not clear that whether psychological stress has a direct effect on atherosclerotic plaque stability. The purpose of this study was to investigate effects of chronic psychological stress on inflammatory marker (ICAM-1) in atherosclerotic plaque, and inflammatory markers in peripheral blood. Materials and methods  Sixty male rabbits were randomized into 2 groups: the control group (n=10) and the atherosclerotic group (n=50). The latter were fed on high fatty diet and were given a large dose of vitamin D₃ (3 600 000 IU/kg) via intraperitoneal injection. After 8 weeks, the atherosclerotic model was established. Then the 50 atherosclerotic model rabbits were divided into 3 subgroups: no-stress subgroup (n=16), physiological stress subgroup (n=16) and psychological stress subgroup (n=18). In physiological stress subgroup and psychological stress subgroup, drinking was cut from twice a day to once a day. At the same time, psychological stress subgroup was given empty bottle stress, and this process lasted for 2 weeks. One hour after the last stress, the blood samples were collected and the serum levels of CRP, IL-6 and ICAM-1 were tested by radioimmunoassay or enzyme linked immunosorbent assay. The aorta and heart were extracted for pathology examination, and the expression of ICAM-1 was tested by immunohistochemical examination.

Results  (1) After effective atherosclerotic animal model construction, the expression of ICAM-1 in aorta was higher in atherosclerotic group than that in control group (P<0.01), and was notably higher in psychological stress subgroup than that in no-stress subgroup or in physiological stress subgroup (2.18±0.15 vs 1.22±0.15, P<0.001, respectively). The expression in physiological stress subgroup was higher than that in no-stress subgroup (58±0.22 vs 1.22±0.15, P=0.001). (2) The serum level of IL-6 (51.80±4.60 pg/ml vs 27.60±4.19 pg/ml) and CRP (1.00±0.37 vs 0.90±0.29 pg/ml) in psychological stress group were significantly higher than that in other groups (All P<0.05). There was a positive relationship between the serum level of CRP, IL-6 and ICAM-1 and the expression of ICAM-1 in aorta wall (r=0.59, r=0.75, r=0.87, P<0.01, respectively). Conclusions  Psychological stress induces an increased expression of ICAM-1 in aortic atherosclerotic plaque, a higher serum level of CRP, IL-6, and sICAM-1 expression. Psychological stress has a direct effect on the transition from stability to unstability through in-plaque and out-plaque inflammation. The serum level of CRP, IL-6 and ICAM-1 can reflect the inflammatory degree in atherosclerotic plaque. (J Geriatr Cardiol 2008; 5:235-242)

Key words  psychological stress; inflammation; atherosclerosis; plaque stability

Introduction

A large body of evidence indicates that psychological stress plays some roles in the etiology and progression of certain cardiovascular diseases, such as atherosclerosis, and in the process of sudden cardiac death, while the substantial etiology of sudden cardiac death was coronary arteriosclerosis disease.¹² Acute coronary syndrome (ACS) is a kind of acute cardiac ischemic syndrome, its clinical manifestations include unstable angina, acute myocardial infarction or sudden cardiac death. It is a serious type of coronary arteriosclerosis disease and could induce sudden cardiac death under the condition of psychological stress.¹³ These events arose our interest to study the relationship of psychological stress, coronary arteriosclerosis disease and ACS.

It is reported that psychological stress promotes acute inflammatory response, leads to an increasing circulating level of C-reactive protein (CRP), IL-6, intercellular adhesion molecule (ICAM)-1, and then participates in the cell infiltrating process of atherosclerosis, promotes the emergence of atherosclerosis, and has an important effect on the onset of coronary artery disease. Accumulating evidence indicates
that atherosclerosis is the result of a prolonged and excessive inflammatory process in the vascular wall.\textsuperscript{2-4}

Now it is clear that plaque rupture, platelet aggregation, and thrombogenesis are the main mechanisms of ACS. The inflammation plays a key role in plaque un-stability.\textsuperscript{5} Furthermore, social-psychic factors (for example, depression, hostility and anxiety) can induce ACS through increasing the release of inflammatory factors and activating the monocyte in plaque, resulting in coronary spasm and thrombosis. These factors promote the progression of ACS.\textsuperscript{14} However, it is not clear whether psychological stresses have a direct effect on atherosclerotic plaque. Thus, elucidating this problem is important in the prevention and the therapy for ACS.

Based on the previous study on stress, inflammation and acute coronary occlusion, the present study was designed to interpret the following points: (1) to observe the direct effect of psychological stress on inflammatory marker of atherosclerotic plaque and to discuss the effect and mechanisms of psychological stress on the incidence of ACS; (2) to assess the correlation and significance of inflammatory markers in peripheral blood and those in atherosclerotic plaque, and investigate the possibility of assessing the plaque stability through the level of peripheral blood inflammatory markers.

Materials and methods

Preparation of arteriosclerosis rabbit model

Sixty male rabbits (3-months-old with 1000-2000g weight) were purchased from Henan Provincial Experimental Animal Center (Zhengzhou, China), acclimatized for 1 week on standard feed and then fed on an experimental diet. The animals were randomized into two groups: normal control group (n =10) and atherosclerosis group (n =50). Normal control group rabbits were fed with common rat chow and normal saline (the same volume of saline was injected intraperitoneally at the beginning of feed). Atherosclerosis group rabbits were fed with high fat diet and vitamin D\textsubscript{3} loading (vitamin D\textsubscript{3} 600 000IU/kg was injected intraperitoneally at the beginning of feed). All animals were fed for 12 weeks. High fat diet composition: 3\% cholesterol, 0.5\% sodium cholate, 0.2\% propylthiouracil, 5\% white sugar, 10\% lard and 81.3\% basic rat chow.

Preparation of psychological stress rabbits model (stimulation of empty bottle)

Fifty atherosclerosis model rabbits were randomized into three subgroups: no stress (n =16), physiological stress (n =16) and psychological stress (n =18) subgroup. Physiological stress model was established with water deficiency in certain time and psychological stress model was established with water deficiency and stimulation of empty bottle for two weeks.

Stress procedure

Control group: vehicle treated control animals were fed with common diet freely and the same volume of saline was injected intraperitoneally, and were fed water twice a day.

Atherosclerosis group was divided into following 3 subgroups:

No stress subgroup: rabbits were fed a high fat diet freely and vitamin D\textsubscript{3}, loading, and were fed water twice a day.

Physiological stress subgroup: rabbits were fed a high fat diet freely with vitamin D\textsubscript{3}, loading, and were fed water twice a day. In the 7-12 weeks, rabbits were fed water once a day. During the water deficiency, rabbits were given no stimulation of empty bottle, which excluded the influence of physiological water deficiency on psychological stress.

Psychological stress subgroup: rabbits were fed a high fat diet freely with vitamin D\textsubscript{3}, loading, and were fed water twice a day. In the 7-12 weeks, rabbits were fed water once a day. During the water deficiency, rabbits were given stimulation of empty bottle. The rabbits were subjected to a broadband noise at 100 db daily for 5s every minute during either a 1- or 3-h period (at random) around midnight, at the height of the diurnal activity cycle. All stressed rabbits were subjected to the same stress schedule. Unstimulated rabbits were exposed only to the normal activity of the animal room.\textsuperscript{8}

Measurement of CRP, IL-6 and sICAM-1

After psychological stress the experimental animal was acclimatized for 1 hour and then anesthetized by being injected intraperitoneally 10\% chloral hydrate. Blood sample was collected from the crossing of the common iliac arteries and standed for 1 hour, then centrifugated for 10 minutes by 3000 rpm. The supernatants were collected, separately loaded and stored at -70\degreeC. The concentrations of CRP, and sICAM-1 were assayed by specific enzyme-linked immunosorbent assay (ELISA) kits. IL-6 levels in the culture supernatants were determined by radio-immunological assay (RIA) kits purchased from Technology Development Center of General Hospital of Chinese PLA.

Histopathologic analyses

All animals were sacrificed at the end of 12 weeks. Study and histologic analysis were performed. The aortas were excised. Tissues were routinely fixed in 4\% buffered paraformaldehyde and embedded in paraffin. After fixation, the aorta was divided into segments of 8-12mm, and incubated in 0.05\% hydrogen peroxide in methanol for 20 min at room temperature. After stained treatment, the expression of sICAM-1 of aorta was estimated using immunohis-tochemical staining. Known positive staining slice from Wuhan Boster Biotechnology Company was treated as positive control. The cytoplasm of sICAM-1 positive endothel-
lial and immune cells was stained brownish yellow and that of sICAM-1 negative control cells was not stained. Macroscopic findings of the aorta were classified into four grades: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

Statistical analysis
All values were expressed as the mean±SEM. Data were compared between the subgroups by using analysis of variance (ANOVA). Correlation between two variables was analyzed with linear correlation. A value of P<0.05 was considered statistically significant.

Results

Confirmation of successfully established model of atherosclerosis in rabbits
After 12 weeks’ feeding, the heart and aorta were removed from the rabbits, weighed, sliced (hearts were cut transversely into at least four biventricular cardiac cross-sections), fixed in 10% Formalin, embedded in paraffin, and stained with HE staining. In the control group, macroscopic examination revealed each layer structure in the vascular wall of arteries was intact. While the pathologic slices of aortic structure in atherosclerotic group showed the characteristic of atherosclerosis, it was presented that the continuity of vascular endothelium was interrupted, and protrusion of local vascular wall to vascular lumina formed plaques, smooth muscular hyperplasy, the disorder arrangement, rupture calcification of elastic fiber in tunica media, thinning of outer membrane, intima injury and calcification in under-intima. Histological examinations of coronary artery revealed proliferation and calcification of vascular smooth muscle cells. The above manifestation suggested the successful atherosclerosis model preparation (Figure 1 A-F).

The influence of psychological stress on the expression of aortic ICAM-1
Figure 2 A-D revealed that ICAM-1 was expressed in the whole slice of non-stress subgroup, physiological stress subgroup and psychological stress subgroup. Hematoxylin staining suggested that the expression of this factor located in the cell membrane and cytoplasm of aortic endothelial cell and some cell of vascular media. There was only less expression of ICAM-1 in the vascular wall of control group animals. Statistical analysis suggested the expression intensity among all the subgroups had significant difference (P<0.01). The expression intensity of ICAM-1 in psychological stress subgroup was significantly higher than those in physiological stress and no-stress subgroups (2.18±0.17 vs 1.58±0.22, 2.18±0.17 vs 1.22±0.15, P<0.001 in both subgroups). While the intensity in physiological stress subgroup was significantly higher than that in no-stress subgroup (1.58±0.22 vs 1.22±0.15, P=0.001), and the expression in no-stress subgroup significantly higher than...
that in normal control group (1.22±0.15 vs 0.13±0.08, P<0.001). See Figure 3A.

**The change in serum CRP, IL-6 and ICAM-1 concentration during the stress**

There were no significant differences of serum CRP levels among normal group, no-stress subgroup and physiological stress subgroup (P>0.05), but those in psychological stress subgroup was significantly higher than those in the other subgroups (P<0.05). The serum concentrations of IL-6 and ICAM-1 in psychological stress subgroup were significantly higher than those in the other subgroups (P<0.05). As compared with non-stress subgroup and normal control group, physiological stress subgroup had significantly higher IL-6 and ICAM-1 concentrations (P<0.001). There was no significant differences of IL-6 and ICAM-1 concentrations between no-stress subgroup and normal control group (P>0.05). See Figure 3B and C.

**Correlation between serum CRP, IL-6 and ICAM-1 concentrations and ICAM-1 expression in aortic plaques during the stress**

As shown in Figure 4 A-C, serum CRP, IL-6 and ICAM-1 concentrations had positive correlation with the expression intensity scores of ICAM-1 in local aortic plaques in atherosclerosis rabbit model. The coefficient correlations were 0.59, 0.87 and 0.75, respectively (P<0.01).
A large body of evidence indicates that psychosocial factors play some role in the etiology and progression of certain cardiovascular diseases such as atherosclerosis. Accumulating evidence also indicates that atherosclerosis is the result of a prolonged and excessive inflammatory process in the vascular wall. Therefore, it is important to inquire whether stressful psychosocial factors can initiate or participate in the inflammatory events that culminate in atherosclerosis. The experiment suggested that psychologi-
cal stress induces a increasing expression of ICAM-1 in aortic atherosclerotic plaque and a higher serum level of CRP, IL-6 and sICAM-1 expression. The expression of ICAM-1 in aorta was positively related to the serum level of CRP, IL-6 and ICAM-1. The serum level of CRP, IL-6 and ICAM-1 can reflex the inflammatory degree in atherosclerotic plaque. Our results indicated psychological stress had a direct effect on the transition from stability to unstability through in-plaque and out-plaque inflammation.

Selection of stress and atherosclerosis model animals

In our experiment, rabbits were selected as atherosclerosis animal model and then administrated with stimulation of empty bottle and water deficiency. In social species, stimuli originate from social and resident environment with well-known impact on neuroendocrine and physiological systems. Social impact and physical affliction, which results from exposure of male rabbits to empty bottle and water deficiency, are the situations frequently met in the natural habitat.9,10 Fear usually develops towards specific adverse stimuli and is the primary response in coping with stress. Fear is also used as a synonym of anxiety in stress-based animal models of depression, where it can be induced through conditioning procedures. Fear conditioning is common to all species studied and has proved a convenient starting point for research on neurobiology of emotions, since it links emotion and memory. The majority of studies of conditioned fear have induced defensive behavior in rabbits by using aversive stimuli, such as loud noise, bright light, and electric shock. Convergent data have demonstrated that a social-aggressive encounter induces physiological evidence of stress, including elevations in plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels, increased heart rate and body temperature, and altered activity of neurotransmitter systems in brain. Chronically water avoidance stressed rabbits also exhibited anxiety-like behaviors, and caused small but significant increases in the mast cell numbers and the expression of IL-1α and IFN-γ. Repeated exposure to water avoidance stress in rabbits could be used as a new chronic stress model. So in our experiment, the basic stimuli of empty bottle and water deficiency offer good validity with anxiety disorders, which in humans are mainly related to social factors and associated with hypothalamic pituitary adrenal (HPA) axis deregulations.9,10

Rabbits’ strong adaptability to new environment and compliance to positive and negative strengthening sensory instruction training made the rabbit as one of the best stress model animals. Rabbits were fed with simple high fat diet, Only hypercholesterolemic animal models were established, and atherosclerotic lesion was not developed. Combining the high fat/cholesterol diets containing sodium cholate and propyl thiouracil with injection of vitamin D₃, led a new way to establish an experiment atherosclerotic model in rabbits.11,12 Therefore, the AS rabbit is an attractive experimental model of the disease as it allows the study of the pathological and pharmacological mechanisms of atherosclerosis.13

Atherosclerosis can be considered to be a modified form of chronic inflammation induced by lipids,14 and an increasing number of studies have focused on the expressions of growth factors and adhesion molecules in atherosclerotic lesions, which are confirmed to play central roles in angiogenesis and endothelial dysfunction.15 Psychological stress promotes acute inflammatory response, leads to an increasing circulating level of CRP, IL-6, ICAM-1, and then participates in the cell infiltrating process of atherosclerosis, and promotes the emergence of atherosclerosis.2,4-6 In this experiment, the animals were administrated effective physiological and psychological stress. Expression of adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule (VCAM)-1, is increased in coronary atherosclerotic tissue and inflammatory cytokines such as IL-1β and TNF-α increased the expression of ICAM-1 and VCAM-1 through the activation of nuclear factor-κ B (NF-κB). The increased expression of cell adhesion molecules is important, since it causes inflammatory cells to be targeted to the injured arteries.16 ICAM-1 is considered an important mediate molecule, which is the induction of specific and reversible cell–cell adhesion, resulting in intercellular communication. The upregulated ICAM-1 expression kinetics have been demonstrated in the formation of the macrophage-derived foam cells. Increased ICAM-1 expression was associated with a marked monocyte and T lymphocyte intimal recruitment. The significantly increased ICAM-1 appeared to be proportional to the extent of atherosclerotic lesion and played more important roles during the early stages of atherogenesis.17 Our results suggested that high fat diet plus injection of vitamin D₃ significantly promoted the expression of ICAM-1 in atherosclerosis plaques locality. The physiological and psychological stress both significantly increased the expression intensity of ICAM-1 comparing with the control group, while psychological stress subgroup had the highest expression intensity among the experiential subgroups. The results indicated the increasing ICAM-1 levels would be the reason of psychological stress inducing atherogenesis. Increasing expression of ICAM-1 could promote the adhesion of Jurkat T cells to IL-1β-activated aortic smooth muscle cells and the development of atherosclerosis.16

CRP is an acute phase reactant synthesized in the liver, and levels are associated with the development of coronary heart disease. Inflammation, manifested by elevated serum levels of CRP is associated with an increased risk of cardiovascular events. Additionally, CRP which has recently emerged as one of the most important inflammatory mediators, can directly participate in the pathogenesis of atherosclerosis by activating endothelial cells and pro-
motivating the inflammatory component of atherosclerosis.\(^1\) Furthermore, CRP has been shown to induce expression of adhesion molecules ICAM-1 in human endothelial cells (ECs). A stepwise increase in the concentration of CRP further enhanced the expression of ICAM-1 in human umbilical vein ECs. The specificity of the proinflammatory effect of CRP on ECs may be inside the cytoplasm of ECs rather than on surface of the EC membrane.\(^2\) In our experiment, high fat diet, following physiological and psychological stress increasing the serum levels of CRP accordingly, the severity degree of atherosclerosis increased in turn, which suggested the inflammatory factor induced by stress could promote the development of atherosclerosis and link with stress and atherosclerosis. The increasing CRP level was correlated with the intensity of ICAM-1, consistent with the previous study that increasing CRP induced expression of ICAM-1, but the mechanisms should be investigated further.

ICAM-1 and CRP concentrations are significantly increased in chronic stable angina pectoris patients with rapid coronary artery disease progression during follow-up compared with those without progression, regardless of plaque morphology. Inflammatory mechanisms involving endothelial and monocyte/macrophage activation appear to play a significant role in atheromatus plaque vulnerability and rapid CAD progression.\(^3\) In the process of atherosclerosis, IL-6, as a pro-inflammatory and procoagulant agent, with potential major implications on atherosclerosis, is an inflammatory cytokine produced by endothelial cells, smooth muscle cells, and macrophages and plays a central role along with TNF-\(\alpha\) in the amplification of the inflammatory cascade progression and thrombotic complications. Rus et al.\(^4\) showed 200-fold higher levels of IL-6 in atherosclerotic arterial walls than in blood.\(^5\) It has been shown that IL-6 stimulates macrophages to secrete monocyte chemotactic protein 1, promotes the expression of adhesion molecules and the secretion of other cytokines by endothelial cells, and the proliferation and the migration of smooth muscle cells. IL-6 is the main hepatic stimulus for CRP production,\(^6\) thus is a major determinant of CRP hepatic synthesis, and leads to a series of procoagulant actions.\(^7\) Meanwhile, IL-6 and CRP are interactive. CRP may contribute directly to the proinflammatory state. CRP stimulates monocyte release of inflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF-\(\alpha\) and may also directly act as a proinflammatory stimulus to phagocytic cells by binding to the FcγRII receptor. CRP also causes expression of ICAM-1 and VCAM-1 by endothelial cells in endothelial cells. IL-6 and serum ICAM-1 levels are positively correlated, and that the association between the IL-6 plasma concentration and atherosclerosis is overlapped by the association between serum ICAM-1 levels and atherosclerosis.\(^8\) In this experiment, the serum level of CRP, IL-6 and ICAM-1 in emotional stress subgroup was significantly higher than those in other subgroups. There was a positive relationship between the serum level of CRP, IL-6 and ICAM-1 and the expressing of ICAM-1 in aorta locality, which reflected the interaction of CRP, IL-1 and ICAM-1 in the development of atherosclerosis promoted by psychological stress. Under different stress conditions, serum IL-6 and TNF-\(\alpha\) concentrations were different. Socioeconomic status differences in levels of circulating IL-6 and TNF-\(\alpha\), interpreted due in part to chronic moderate inflammation and central nervous system-stimulated activation of stress pathways. The CRP results are consistent with this pattern, since IL-6 and TNF-\(\alpha\) are important determinants of CRP in our studies.\(^9\)

So, possible pathways of stress promoting the development of atherosclerosis are as follows: psychological stress resulted in sympathetic nerve excitation and promoted the catecholamine secretion, which directly activated immunocyte and produce immunologic factors, then promoted the expression of ICAM-1 and other cytokines. Cytokines infiltrated into atherosclerosis plaque locality, made plaque unstability, rupture and thrombosis and promoted the occurrence of acute coronary syndroms. Psychological stress had a direct effect on the transition from stability to unstability through in-plaque and out-plaque inflammation. But the detailed mechanisms need further investigation.

**References**