Original Article
Excessive RAAS, ER stress and inflammation responses impair adaptability to acceleration-associated stress in swines with moderate-severe coronary artery stenosis

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Abstract: It is reported that there is higher morbidity of coronary heart disease in pilots than in the general population. During flight, pilots always suffer from acceleration-associated stress (+Gz stress). There have been few studies about the adaptability to +Gz stress in pilots with coronary heart disease. Coronary artery stenosis (CAS) model was established by ligation of proximal left anterior descending branch of heart in swines. A trapezoid acceleration curve was used for +Gz stress. ELISA was used to analyze RAAS factors and inflammatory factors in plasma. Immunoblotting was applied to determine myocardial GRP78 levels. The maximum tolerated +Gz values in the moderate and severe CAS group were both less than the mild CAS and sham-operated group. The expression levels of RAAS (Ang II and ALD) factors and inflammatory factors (CRP, TNF-α, SAA, sPLA2, MMP-1, MMP-2 and MMP-3) were significantly higher in moderate and severe CAS groups than mild CAS and sham-operated groups before and after +Gz stress. The GRP78 expression levels were significantly increased in moderate and severe CAS groups than mild CAS and sham-operated groups after +Gz stress. These results show that the adaptability to +Gz stress in swines with moderate and severe CAS is decreased significantly. Excessive RAAS, ER stress and inflammatory responses are detrimental to the tolerance in swines to +Gz stress with moderate and severe coronary artery stenosis.

Keywords: Coronary artery stenosis, acceleration-associated stress, renin-angiotensin-aldosterone system, Endoplasmic Reticulum Stress, inflammation

Introduction

During flight, pilots suffer from detrimental changes of their work environment, such as acceleration-associated stress (+Gz stress), noise, radiations, heat, hypoxia and vibration. All these factors can cause cardiac structural damages [1-3], and increase the risk of coronary heart diseases (CHDs) [4]. It is reported that coronary heart disease may occur in pilots 3-15 years younger than in the general population and that fighter pilots had the highest rates of acute myocardial infarction and chronic ischemic heart disease [5]. In the search for the advantage in aerial combat, increasing higher performance aircrafts that have been developed impose ever greater acceleration force loads on the pilots [6]. The adaptation of human body during flight is very important because it decides the healthy condition and performance of pilots, and the safety of flights. In the present study, we used a swine model with mild, moderate, and severe coronary artery stenosis (CAS) to detect how the severity of coronary artery stenosis affects the tolerance level to +Gz stress in swines.

As we know, stress can activate renin-angiotensin-aldosterone system (RAAS). Appropriate activation of RAAS under stress is a protective reaction in human body. However, excessive activation of RAAS has adverse effects on multiple organs, including heart and blood vessels [7-9]. Angiotensin II (Ang II) and aldosterone (ALD), are vasoconstrictive peptides and are involved in the development of coronary heart disease [10, 11]. According to recent studies, RAAS can induce or mediate Endoplasmic Reticulum Stress (ER stress) [12, 13]. ER stress-induced unfolded protein reaction (UPR) signal-
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Glucose-regulated protein 78 (GRP78) is the main UPR regulator [15]. UPR is associated with the production of many pro-inflammatory molecules, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), serum amyloid A (SAA), and secretory phospholipase A2 (sPLA2) [16-18]. These pro-inflammatory molecules are called acute phase proteins (APPs) [14]. APPs can aggravate tissue damage and cause more severe sterile immunopathological conditions [19, 20]. Matrix metalloproteinases (MMPs) are inflammation factors that may facilitate atherosclerosis and plaque destabilization [21, 22]. Clinical studies support that MMPs play an important role in the development of acute coronary syndromes [23, 24].

The purpose of the present study is to test the hypotheses that excessive RAAS activation, ER stress and inflammation responses impair the tolerance to acceleration-associated stress in swines with moderate and severe CAS.

Materials and methods

Animals

Twenty-five healthy male Bama miniature swine (10.0±1.6 months old, 23±2 kg) were obtained from the Heilongjiang Shuangyashan Miniature Swine Farm. Swines were raised in the Animal Center of the Fuwai Cardiovascular Hospital (under the license: SYXK (Beijing) 2008-0016). Swines were fed twice daily with grain according to the animals’ growth requirements using a normal diet, and were allowed free access to drinking water. Their activity, gait, secretions, food and water uptake, urine, stool and body weight were recorded daily before surgery. The animals used in this study has been obtained, cared for, and used in accordance with the Animal Welfare Act and the “Guide for the Care and Use of Laboratory Animals” from the Institute of Laboratory Animal Resources in China. This study was approved by the Animal Welfare and Ethics Committee of Fuwai Cardiovascular Hospital (license #2010-1-20-125ZD).

Coronary artery stenosis model by thoracoscopy

After a 24-h fasting, twenty swines were anesthetized with intramuscular ketamine (35 mg/kg) and diazepam (1 mg/kg) and placed in a right lateral position. Breathing was supported using a Savina intensive care breathing machine (Dräger, Lubeck, Germany). Pericardium was cut 2 cm from the diaphragmatic nerve, exposing the heart. The left anterior descending (LAD) artery, and its forward diagonal and ventricular branches were identified. The LAD artery was gently separated using forceps in its proximal diagonal branch, 1 cm apart from the left main branch. At the proximal LAD artery bifurcation, casing needles of different sizes were placed and the blood vessels were ligatured with silk suture. The casing needles were gently withdrawn, thus controlling the degree of narrowing. Following the surgical procedure, each swine underwent a quantitative computerized angiography (QCA) for confirmation of the degree of stenosis using an OEC9800 digital subtraction angiography machine (GE Healthcare, Waukesha, Wisconsin, USA) [25]. Using conventional projection angles, the continuous movie method was used to observe the degree of LAD artery stenosis. QCA was performed by the same group of three experienced professional cardiac interventional physicians using the VisionRis quantitative analysis software (Beijing Weiye Future Technology Co., Ltd., Beijing, China) [26]. Vessel diameter of the target lesion and reference vascular diameter were then collected. The reference vascular diameter was the diameter of the normal vessel proximal to the lesion. The percentage of stenosis was calculated as [(1-(vessel diameter of the target lesion/reference vascular diameter)) ×100%].

According to their CAS degree, swines were divided into three groups: mild (stenosis of 20-50% of the reference vascular diameter, n=7), moderate (stenosis of 51-70%, n=6), and severe (stenosis of 71-90%, n=5) [27]. Two swines were euthanized at 8 and 48 h after surgery in the severe stenosis group because they suffered acute myocardial infarction. Five swine underwent a sham surgery using identical anesthetic and surgical procedures, but without LAD artery ligation.

Animals were kept for 1 week under standard diet before they were exposed to +Gz stress. During this time, these animals received low molecular heparin calcium (2100 IU) at 6 h after surgery. Clopidogrel (75 mg/day), aspirin (100 mg/day) and penicillin (4.8 million units/day for 3 days) were administrated every 12 hours.
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Grouping, acceleration-associated stress (+Gz stress) exposure and specimen collection

The type 98 centrifuge (China Astronaut Research Training Center Development) is equipped with an 8-meter arm, and can produce a G force ranging from 1.41 to 16 G, with a maximum rate of 6 G/s. The single-pod uniaxial cabin has an effective carrying capacity of 165 kg. For all centrifuge procedures, swine were placed in a form-fitted aluminum and fibreglass restraint system (barrel with a soft cushion filling) (80 cm tall and 35 cm in diameter) and held in place with straps behind the head, at mid-chest, and across the hips. The restraint system positioned the swine in a normal standing position with the weight of the animal supported along the ventral surface. The head was facing the centrifuge rotation axis. Therefore, the inertial load (+Gz stress) was parallel to the spine in a head-to-toe direction, similar to the +Gz stress exposure of pilots of high-performance aircrafts. Before being placed in the restraint chair, swine were anesthetized with ketamine (10 mg/kg) and diazepam (1 mg/kg) by intramuscular injection. Then, 5-lead (chest) ECG leads were placed. Swine were then exposed to +Gz stress. They first experienced a +3 Gz stress for 10 s, and a trapezoid acceleration curve was used after 5-10 min. Beginning with an initial +3 Gz stress exposure, the G-value was increased at a rate of 1 G/s, for 60 s, for each exposure. During the +3 to +9 Gz exposure, if the ECG monitor displayed risk signals (the same lead showing successive premature ventricular contraction three times or more, or ventricular fibrillation), the +Gz stress exposure would be immediately stopped. Intervals between exposures were 10 min, and the maximal +Gz stress was +9 Gz. ECG and physiological parameters were continuously recorded using a FE-30 magnetic recorder (Sony, Tokyo, Japan). Plasma was collected before and after +Gz stress exposure and myocardial tissue was collected after +Gz stress exposure.

ELISA

ELISA kits (Westang Bio-tech Co. Ltd, Shanghai, China) were utilized to quantify RAAS factors (Ang II and ALD) and inflammatory factors (CRP, TNF-α, SAASPLA2, MMP-1, MMP-2 and MMP-3) in plasma. Samples and standards were prepared according to manufacturer’s instructions. Absorbance of standards and samples were determined spectrophotometrically at 450 nm, using a microplate reader (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Results were plotted against the linear portion of a standard curve.

Immunoblotting

Immunoblotting was applied to determine myocardial GRP78 levels. Protein samples were...
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Table 1. Maximal tolerated +Gz values were decreased in moderate and severe CAS groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham-operated group (n=5)</th>
<th>Mild CAS group (n=7)</th>
<th>Moderate CAS group (n=6)</th>
<th>Severe CAS group (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal tolerated +Gz values</td>
<td>8.0±0.7</td>
<td>7.7±1.1</td>
<td>6.0±0.9*#</td>
<td>5.2±0.8*#</td>
</tr>
</tbody>
</table>

*P<0.05 versus the Sham-operated group, *P<0.05 versus the Mild stenosis group.

Separated on gradient (4%-20%) mini-gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% skim milk solution for 1 hour at room temperature. The blocked membranes were incubated with a primary antibody against a protein of interest. After washing with phosphate-buffered saline containing 0.05% Tween20, the membranes were incubated with a peroxidase-linked secondary antibody specific to the primary antibody applied. After further wash, membranes were treated with enhanced chemiluminescence reagents. The membranes were exposed on x-ray films. Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD) was used to analyze band density.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, Illinois, USA). Analysis of variance (ANOVA) with Fisher post-hoc test was used to analyze differences between experimental groups, and differences were confirmed using the Mann-Whitney U-test. Statistical significance was defined as P<0.05.

Results

Maximal tolerated +Gz values were decreased in swines with moderate and severe CAS

A total of 23 swines with different coronary artery stenosis underwent the centrifuge test (Figure 1). The +Gz stress was considered as the maximum tolerated +Gz stress when ECG monitoring displayed risk signals. In this study, we found that there was no difference between the mild CAS group and sham-operated group in maximal tolerated +Gz values. However, the maximum tolerated +Gz values in the moderate CAS group and severe CAS group were both less than the mild CAS group and sham-operated group (Table 1).

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In the present study, we measured the levels of Angiotensin II (Ang II) and aldosterone (ALD) in plasma before and after +Gz stress exposure. We found that there were higher levels of Ang II and ALD in moderate and severe ACS groups than sham-operated and mild ACS groups in baseline. +Gz stress increased the activation levels of Ang II and ALD in mild, moderate and severe CAS groups. In moderate and severe CAS groups, Ang II and ALD levels were still higher than the levels in the mild CAS group and sham-operated group after +Gz stress exposure. There were no difference in Ang II and ALD levels between mild CAS group and sham-operated group before and after +Gz stress exposure (Table 2). Thus, there were augmented activation of RAAS in swines with moderate and severe CAS before and after +Gz stress exposure.

There were augmented inflammatory responses in swines with moderate and severe CAS before and after +Gz stress exposure

In the present study, we measured the expression levels of acute phase proteins (APPs), including CRP, TNF-α, SAA and sPLA2 in plasma. The expression levels of matrix metalloproteinases (MMPs) in plasma, including MMP-1, MMP-2 and MMP-3, were determined as well. In moderate and severe CAS groups, APPs and MMPs levels were both higher than the levels in the mild CAS group and sham-operated group before and after +Gz stress exposure (Figures 2, 3). We found that +Gz stress increased the expression levels of APPs and MMPs in all the groups. There were no differences in APPs and MMPs levels between mild CAS group and sham-operated group before and after +Gz stress exposure. Thus, there were augmented inflammatory responses in swines with moderate and severe CAS before and after +Gz stress exposure.
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Table 2. Activity of RAAS is augmented in swines with moderate and severe CAS

<table>
<thead>
<tr>
<th>Groups/RAAS factors</th>
<th>Before +Gz</th>
<th>After +Gz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham-operated group (n=5)</td>
<td>Mild CAS group (n=7)</td>
</tr>
<tr>
<td>Ang II (ng/ml)</td>
<td>1.7±0.3</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>ALD (pg/ml)</td>
<td>474.7±67.3</td>
<td>527.8±244.6</td>
</tr>
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</table>

*P<0.05 versus sham-operated group, \&P<0.05 versus mild stenosis group, *P<0.05 versus before exposure in the same group.
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Figure 2. Expression levels of acute phase proteins (APPs) are augmented in swines with moderate and severe coronary artery stenosis before and after +Gz stress exposure. *P<0.05 versus sham-operated group, †P<0.05 versus mild stenosis group, ‡P<0.05 versus before exposure in the same group.

Figure 3. Expression levels of matrix metalloproteinases (MMPs) are augmented in swines with moderate and severe coronary artery stenosis before and after +Gz stress exposure. *P<0.05 versus sham-operated group, †P<0.05 versus mild stenosis group, ‡P<0.05 versus before exposure in the same group.
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There were increased expression levels of myocardium GRP78 in swines with moderate and severe CAS after +Gz stress exposure

Glucose-regulated protein 78 (GRP78) is the main UPR regulator [15]. We tested GRP78 expression in myocardium tissue. The results showed that expression levels of GRP78 were higher in moderate and severe CAS groups than the levels in sham-operated and mild stenosis groups after +Gz stress (Figure 4).

Discussion

It is reported that there is higher morbidity of coronary heart disease in pilots than in the general population [5]. During flight, pilots always suffer from +Gz stress. And +Gz stress is a strong stress that may cause cardiac urgent events and affect the safety of pilots and flight seriously. So it is very important to study the mechanisms of human body's adaptability to +Gz stress in pilots with coronary heart disease. There haven't been studies about RAAS, ER stress and inflammation responses in the research of adaptability to +Gz stress in coronary heart disease objects.

In the study, we found that adaptability to +Gz stress in moderate and severe CAS groups is decreased significantly. The expression levels of RASS factors (Ang II and ALD) and inflammatory factors (CRP, TNF-α, SAA, sPLA2, MMP-1, MMP-2 and MMP-3) were significantly higher in moderate and severe CAS groups than mild CAS and sham-operated groups before and after +Gz stress. The GRP78 expression levels were significantly increased in moderate and severe CAS groups than mild CAS and sham-operated groups after +Gz stress.

As we know, stress can activate RAAS and +Gz stress is a large stress to human body. RAAS play an import role in the development of cardiovascular disease. Two key neurohormones within the RAAS, Ang II and ALD, are crucially involved in the pathology of artherosclerosis, and also involved in mediating adverse effects on the cardiovascular system both systemically and within tissues [10, 11, 28]. We detected the levels of Ang II and ALD in plasma and found there were augmented activation of RAAS in swines with moderate and severe CAS. The augmented RAAS activation can promote the rupture of plaque and induce myocardium ischemia and acute heart failure [29, 30]. As a result, the excessive activation of RAAS may impair the adaptability of +Gz stress in swines with moderate and severe CAS.

According to recent studies, RAAS can induce or mediate ER stress [12, 13]. ER stress-induced UPR signaling. Glucose-regulated protein 78 (GRP78) is the main UPR regulator. UPR is associated with the production of many pro-inflammatory molecules, called acute phase proteins (APPs) [14, 19]. APPs can aggravate tissue damage and cause more severe sterile immunopathological conditions [20, 31]. APPs play an important role in the development of atherosclerosis and cardiovascular diseases [20, 32, 33]. We tested the expression levels of GRP78 and some APPs, and found there were higher levels of GRP78 and APPs in moderate and severe CAS groups than mild CAS group and sham-operated group. These results indicate that excessive activation of RAAS induces excessive ER stress, then excessive ER stress induces excessive acute phase responses in moderate and severe CAS groups, that may aggravate coronary heart disease and impair the adaptability of +Gz stress in swines with moderate and severe CAS.

In the study, we found that there were higher expression levels of MMP-1, MMP-2 and MMP-3.
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in moderate and severe CAS groups than in mild CAS and sham-operated groups before and after +Gz stress exposure. Matrix metalloproteinases (MMPs) are inflammation factors that facilitate atherosclerosis, plaque destabilization and play an important role in the development of acute coronary syndromes [21, 22]. Circulating MMP levels are elevated in patients with acute myocardial infarction and unstable angina [23, 24]. According to our results, excessive expressions of MMPs may be one of the mechanisms of low adaptability to +Gz stress in swines with moderate and severe CAS.

Altogether, excessive RAAS, ER stress and inflammation responses can contribute to development of atherosclerosis, myocardium ischemia, plaque destabilization or rupture, heart failure and malignant arrhythmia and so on [20-24, 29, 32, 33]. These may be important mechanisms of low adaptability to +Gz stress in swines with moderate and severe CAS.

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Disclosure of conflict of interest

None.

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