Myocardial ischemia-reperfusion injury is enhanced in a model of systemic allergy and asthma

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MATERIALS AND METHODS

Guidelines for animal research. All animal procedures followed in this study were approved by the East Carolina University Committee on the Use and Care of Laboratory animals and conformed to the standards in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Sensitization of animals. Allergic sensitization of New Zealand White rabbits was performed using protocols well established in our laboratory.
laboratory, as originally described by Ali and Metzger (1). Rabbits were sensitized by intraperitoneal injections of allergen adsorbed to Alum (312 arbitrary units of Dermatophagoides pteronyssinus and Dermatophagoides farinae extract, Greer Laboratories, Lenoir, NC; Imject Alum, Pierce; Rockford, IL). Starting at 24 h postnatal, injections were given at 1-wk intervals for the first month and then at 1-mo intervals. Animals were used for cardiac I/R injury studies at 4–6 mo of age. A subgroup of the sensitized animals was challenged with aerosolized dust mite 7 days after an intraperitoneal injection to induce pulmonary inflammation. A separate group of sensitized and airway-challenged animals was used to determine the titer of serum dust mite-specific IgG and IgE and to evaluate bronchoalveolar lavage (BAL) cell counts.

**BAL cell counts.** BAL was done before and 48 h after challenging animals with aerosolized dust mite. A polyethylene-190 catheter was advanced through the endotracheal tube into the lungs and gently wedged into an airway. The segment beyond the tip of the catheter was lavaged with 2.5 ml of sterile saline, which was collected by gentle aspiration. Total cell counts were determined using a Coulter counter (Beckman Coulter Instruments; Miami, FL). Cytospin preparations were prepared and stained with DiffQuik (VWR Scientific; S. Plainfield, NJ). Differential cell counts were acquired by counting 200 cells per sample.

**Serum IgE titer.** Blood was collected before and on alternate days after intraperitoneal injection until 7 days and before and on alternate days after airway challenge until day 6. Serum levels of dust mite-specific IgG were determined by standard ELISA using peroxidase-conjugated affinity-purified F(ab′)2 fragment donkey anti-rabbit IgG (Jackson ImmunoResearch).

**Serum IgE levels.** Serum levels of IgE were determined semiquantitatively by a percutaneous antigen challenge (PCA) test. Serum (0.2 ml) from sensitized animals was injected intradermally into the clean-shaven back of a naïve rabbit. Serum from unsensitized animals and normal saline were used as control injections. Each sample was injected in different dilutions (1:40, 1:20, 1:10, and full serum) and into two different naïve hosts to exclude the possibility of difference in host response. After 72 h of intradermal injection, each host rabbit was given intravenous injection of 1 ml of solution containing 4% Evans blue dye, 0.9% saline, and 10,000 arbitrary units of dust mite. The skin lesions were traced after 30 min and analyzed by NIH Image analyzer.

**Peripheral blood leukocyte count.** Blood was collected from the ear vein of all animals before I/R at each time point. Total leukocyte counts in blood were determined with the use of a Coulter counter. The smears were prepared and stained with DiffQuik. Differential cell counts were acquired by counting 200 cells per sample.

**I/R injury.** I/R injury was induced in four experimental groups and one control group. In the experimental groups, in situ myocardial I/R injury was created 24 h (1DB) and 7 days (7DB) after the last intraperitoneal boost injection (allergic sensitization groups) and 24 h (1DAWCH) and 7 days (7DAWCH) after an airway challenge (sensitized and airway-challenged groups). Unsensitized animals served as controls. All animals were anesthetized (ketamine 90 mg/ml; xylazine 20 mg/ml; mixed 9:1, administered at the dose of 0.4 ml/kg subcutaneously) and mechanically ventilated with room air (Harvard respirator). A polyethylene cannula was inserted into the right common carotid artery and used to monitor mean arterial pressure. The left common jugular vein was cannulated and used to deliver supplemental anesthesia and fluids to account for insensible fluid loss. After an equilibrium period of 15 min, a thoracotomy was performed. The chest was opened by a transverse incision in the left fourth intercostal space. The ribs were retracted by a rib retractor and the pericardium gently separated. Ligation of a branch of the left anterior descending coronary artery was performed with the use of a 6-0 silk suture (Ethicon) and a reversible snare. Effective occlusion was established by an acute decrease in mean arterial pressure and cyanosis of the distal myocardial tissue. After 30 min of occlusion, the snare was released and the myocardium was reperfused for 3 h.

**Determination and quantification of infarct size.** At the end of 3 h of reperfusion, animals were euthanized and the hearts were excised. The left anterior descending coronary artery was cannulated at the occluded site and perfused with 1.0% 2,3,5-triphenyltetrazolium chloride solution to delineate the infarcted area. The ligature was then tightened to reocclude the artery and 1% Evans blue dye was retrogradely infused through the aorta to demarcate the area at risk. The left ventricles (LV) were separated from the right ventricular free wall and multiple transverse sections were made from each LV and photographed. Area of the LV, the area at risk, and the infarcted area of each section were determined by NIH Image analyzer. The area at risk was expressed as a percentage of the LV area and the area of the infarcted zone was expressed as a percentage of the area at risk.

**Myocardial histology.** Multiple paraformaldehyde (10%) fixed sections were made from each ventricle after the I/R protocol. Sections were embedded in paraffin and 5 µm sections were cut and stained with Gill’s hematoxylin and eosin. The slides were examined by light microscope using high magnification (×40). The number of neutrophils per high-power field was counted; the area under each high-power field was calculated based on the microscope specifications and neutrophil count expressed as the number of neutrophils per square millimeter of tissue. Neutrophil count was made on three sections per animal (n = 3 per group) and five high-power fields per section.

**Statistics.** Data are expressed as means ± SE. Differences between groups were determined by ANOVA and Fisher’s post hoc test (P < 0.05). Pearson’s correlation coefficient was used to determine the significance for linear regression analysis. Paired t-test was used to compare individual cell types before and after treatment.

## RESULTS

**Serum levels of dust-mite-specific IgG.** In sensitized animals, both intraperitoneal injection of allergen adsorbed to adjuvant and exposure of the airways to aeroallergen caused an increase in serum dust mite-specific IgG after 48 h. Serum from control unsensitized animals did not show any change in the IgG titre at similar time points. The preboost level of dust-mite-specific IgG was higher in the sensitized animals than the baseline level in the unsensitized control group. The IgG levels at day 7 after intraperitoneal boost and at all days after airway challenge were significantly higher than the preboost levels (Fig. 1, n = 6 per group; P < 0.05).

**Serum levels of dust-mite-specific IgE.** Serum levels of dust-mite-specific IgE were semiquantitatively assessed by measuring the size of skin lesions at different serum dilutions in a PCA test. Lesions were measured in two separate naïve hosts to eliminate the possibility of host-specific reactions. Lesions caused by serum from sensitized animals (n = 3) at higher concentrations were significantly larger than those caused by control serum or normal saline. There was no difference in the lesion size in both hosts, eliminating the possibility of host-specific reactions to the sensitized sera (Fig. 2; P < 0.01).

**Differential cell counts of BAL fluid.** Differential cell counts of BAL fluid were done before airway challenge and 48 h after airway challenge (n = 6 at each time point). There was a 20-fold increase in eosinophil count (2.5 ± 1.5 × 104 preairway challenge vs. 51.8 ± 2 × 104 postchallenge) after airway challenge, thereby providing evidence that airway inflammation had been induced.

**Differential cell counts in peripheral blood.** Differential cell counts in blood were done at 24 h and at 7 days after an intraperitoneal boost and 24 h and 7 days after an airway challenge, thereby providing evidence that airway inflammation had been induced.

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challenge \((n = 6\) in each group\). At 1DB and 1DAWCH, neutrophil and eosinophil percentages were significantly higher than those before the allergen boost. The increase in peripheral blood neutrophil and eosinophil percentages after airway challenge indicates that sensitization and airway challenge with allergen induce a systemic response in addition to the local airway response \((\text{Fig. 3, A-E})\).

**Myocardial area at risk.** The myocardial area at risk after an I/R protocol was determined by NIH Image analyzer and normalized to the LV area. There was no significant difference in the myocardial risk area across the groups \((n = 6\) per group, Fig. 4A).

**Myocardial infarct size.** The myocardial infarct sizes were determined after the I/R protocol and normalized to the area at risk. In all sensitized groups, the myocardial infarct sizes were significantly greater compared with those in control animals at all time points examined \((1DB, 31 \pm 4; 7DB, 28.9 \pm 2.6; 1DAWCH, 66.1 \pm 4.1; 7DAWCH 28.9 \pm 9.2; \text{control}, 16.7 \pm 3.2; P < 0.01\) by ANOVA; \(n = 6\) in all groups). In addition, the infarct sizes at 24 h postairway challenge were significantly higher than those of other sensitized groups \((P < 0.05\) by ANOVA). These results indicate that systemic allergic inflammation predisposes to the risk of myocardial I/R and the risk is enhanced in presence of acute airway inflammation. The further increase in infarct size at 24 h after airway challenge could be due to an added systemic inflammatory response 7DB. However, the possibility of complications from hypoxia and pulmonary vasoconstriction cannot be eliminated from this study \((\text{Fig. 4B})\).

**Tissue neutrophil count.** A neutrophil count was made on three sections per animal \((n = 3\) per group) and five high-power fields per section. In sensitized animals, the myocardial area at risk during I/R had a higher number of tissue neutrophils compared with the myocardium of control animals with I/R. The tissue neutrophil count expressed as neutrophils per square millimeter of tissue at risk was significantly higher at 1DAWCH and 7DAWCH and at 1DB compared with those in the control group \((P < 0.01\) ). The neutrophil counts at 7DB also had a tendency to be higher than those in the control group, although the difference did not reach the level of statistical significance. In addition, the tissue neutrophil count at 1DAWCH was significantly higher than that after intraperitoneal boost \((P = 0.02; \text{Fig. 5})\).

**Myocardial neutrophil count associated with infarct size.** A correlation analysis revealed a significant positive correlation between tissue neutrophil count and myocardial infarct size \((r^2 = 0.9, P < 0.01; \text{Fig. 6A})\). Although there was an association between the peripheral blood neutrophil percent and infarct size, the correlation was not significant \((r^2 = 0.57, P < 0.01; \text{Fig. 6B})\). Blood neutrophil percent had no correlation to tissue neutrophil count \((r^2 = 0.46; \text{Fig. 6C})\), thereby suggesting that the increase in tissue neutrophil infiltration is not due an increase in blood neutrophil but due to increased recruitment of neutrophils to the site.

**DISCUSSION**

In summary, results of this study indicate that the risk of acute myocardial I/R injury is enhanced in presence of systemic allergic inflammation, and the risk is further increased by acute allergic airway inflammation. Neutrophils have long been implicated in the causation of myocardial damage in I/R. This study showed that during reperfusion, the influx of neutrophils to the myocardial area at risk is enhanced in the presence of background systemic inflammation. The tissue neutrophil count had a strong positive correlation to the myocardial infarct size, thereby suggesting the possibility that increased neutrophil infiltration is a factor responsible for the enhanced tissue damage. In addition, the absence of significant correlation between blood neutrophil percent and tissue neutrophil count suggests that local tissue changes in the myocardium such as expression of increased cell adhesion molecules

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**Fig. 1.** Serum titer of dust mite-specific IgG. A set of sensitized and airway-challenged animals separate from those used for ischemia-reperfusion I/R \((n = 6)\) was used to determine the titer of serum dust mite-specific IgG. Closed circles represent the IgG titer of control serum, whereas open circles represent the IgG titer of sensitized serum. Serum from sensitized animals showed a gradual increase in IgG level after an intraperitoneal (IP) boost. An airway challenge with antigen was done at day 7 of IP boost. After airway challenge, serum IgG showed a slight increase until day 13. Serum from control animals did not show any change in the IgG titer at similar time points. The preboost level of dust mite-specific IgG was higher in the sensitized than the baseline level in controls. The IgG levels at day 7 of IP boost and at all days after airway challenge were significantly higher than the preboost levels. *\(P < 0.05\), significant difference from preboost levels.

**Fig. 2.** Skin lesion size for percutaneous antigen challenge (PCA) test. Rabbit 1 and 2 represent two different host rabbits on which the serum from sensitized rabbits \((n = 3)\) was injected at different concentrations. C, size of skin lesion when control serum from nonsensitized rabbits was injected; NS, size of skin lesion when normal saline was injected; FS, full sensitized serum. Skin lesions induced by serum from sensitized animals at higher concentrations were significantly higher in both host rabbits from those induced by control serum and normal saline. Identical response of both host rabbits excludes the possibility of host variation in the response. *\(P < 0.01\), significant difference from lesions induced by control serum and normal saline.
in the coronary microvasculature and myocardium, increased expression of leukocyte chemotactic factors, such as IL-8, or increased neutrophil activation are responsible for the increased tissue infiltration. Our observation is in accordance with recent observation by Bao et al. (2) that granulocytes from allergic asthmatic subjects show an increased expression of P-selectin glycoprotein ligand-1, which enhances the capability of neutrophils and eosinophils to roll and adhere to inflamed endothelium.

Cardiovascular complications are relatively common in cases of bronchial asthma (5–7, 9, 15, 22, 26, 32). For example, myocardial contractile bands have been described in children that died from bronchial asthma, suggesting the contribution of myocardial pathologies to the mortality (9). Drugs that are currently used in treating asthma, such as theophylline, catecholamines, and inhaled β-agonists have been shown to cause cardiotoxicity (5, 25, 32). However, the therapeutic dose of β-agonists received by most asthmatic patients does not have any significant impact on cardiovascular parameters, suggesting the role of airway-selective β-blockers unlikely in the causation of the cardiovascular complications (30). In the cases observed by Drislane et al. (9), a section of the patients developing myocardial contractile bands did not receive any catecholamines, thereby suggesting catecholamine therapy as an unlikely cause for the observed myocardial pathology. Theophylline has been known to cause cardiac toxicity, such as cardiac arrhythmias, but most complications manifest only at high plasma levels of the drug (33, 34). These high plasma levels commonly occur only in cases of bolus intravenous administration of the drug in the management of status asthmaticus, in cases of reduced metabolism such as the elderly or in cases of overmedication. In addition to potential side effects of drugs used in the treatment of asthma, histamine induced coronary vasospasm (15) and cardiac arrhythmias (8, 21, 31), hypoxia, and pulmonary vasoconstriction secondary to airway obstruction may alter cardiovascular function. Evidence from our study indicates that the asthmatic pathophysiology itself may predispose an individual to cardiovascular dysfunction.
It has been well documented that asthmatic airways and pulmonary vascular endothelium in asthma show upregulation of different cell adhesion molecules essential for the influx of inflammatory cells, which are partly responsible for the pathology and symptoms of asthma (14, 24, 37). However, the effect of chronic inflammation in asthma on the coronary vasculature and the myocardium has not been investigated. In addition, several cytokine levels have been shown to be elevated in asthmatic subjects in BAL as well as in blood (19, 28).

Fig. 4. A: myocardial area at risk of the different groups after I/R injury normalized to the left ventricular area. There was no significant difference in the area at risk across the groups (n = 6 per group). B: infarct sizes of the different groups after I/R injury. Infarct size calculated as a percentage of the area at risk. All experimental groups showed a significantly higher infarct size compared with the control (nonsensitized) group (*P < 0.01). The 24-h postairway challenge group showed a significant increase in infarct size compared with all other groups ($P < 0.05). 1DB, I/R 1 day after intraperitoneal boost; 7DB, I/R 7 days after intraperitoneal boost; 1D AWCH, I/R 1 day after airway challenge; 7D AWCH, I/R 7 days after airway challenge; C, I/R on control (nonsensitized group); n = 6 in all groups.

Fig. 5. Neutrophil count was made on three sections per animal (n = 3 per group) and five high-power fields per section. The myocardial areas at risk in sensitized animals showed a greater tissue infiltration of neutrophils than those in control animals. The tissue neutrophil count expressed as neutrophils per square millimeter of tissue at risk was significantly higher at 1D AWCH and 7D AWCH and at 1DB compared with those in control (*P < 0.01). In addition, tissue neutrophil count in the 1DAWCH group was significantly higher than that of the postboost groups ($P = 0.02).

Fig. 6. A: significant positive correlation between tissue neutrophil count and myocardial infarct size ($r^2 = 0.9, P < 0.01$), thereby suggesting a strong association between tissue neutrophil infiltration and myocardial infarct size. B: there was an association between peripheral blood neutrophil percent and infarct size, but the correlation was not significant ($r^2 = 0.57, P > 0.01$). C: blood neutrophil percent had no correlation to tissue neutrophil count ($r^2 = 0.46$). This suggests that the increase in tissue neutrophil infiltration is not due to an increase in blood neutrophil but due to increased recruitment of neutrophils to the area at risk.
Cytokines such as IL-1β, IL-6, and TNF-α have been shown to have direct toxic effect on cardiomyocytes (4, 18) and alter parameters for leukocyte recruitment during I/R (11). Therefore, it can be inferred that the underlying inflammation in bronchial asthma might account for some cellular changes in circulation and the myocardium that leads to the different cardiac pathologies. Acute I/R injury of the myocardium is a good cardiovascular model to evaluate the inflammatory effects of asthma on the myocardium within a short period of time. Different cell adhesion molecules are also upregulated during ischemia, which helps in recruitment of potentially damaging leukocytes form the circulation during reperfusion.

Considering the parallels in the underlying pathophysiology, if the underlying inflammatory pathology in asthma alters cardiac function, it would be most obvious in case of myocardial I/R injury.

In conclusion, results from this study suggest that in rabbit models, presence of systemic allergic inflammation and allergic asthma enhances the risk of myocardial I/R.

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GRANTS

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