Acute stress induces cardiac mast cell activation and histamine release, effects that are increased in Apolipoprotein E knockout mice

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Abstract

Objectives: Cardiac mast cells have recently been found to be activated in atherosclerotic coronary arteries, but no mediator has so far been documented to be released from them, nor have they been investigated in Apolipoprotein (Apo) E knockout (k/o) mice that develop atherosclerosis. Psychological stress triggers acute coronary syndrome, while acute restraint stress stimulates rat cardiac mast cells, the main mediator of which histamine is a coronary constrictor. Here, we investigated the effect of acute stress on the activation of cardiac mast cells morphologically, as well as the levels of cardiac and serum histamine in normal and genetically deficient mice.

Methods: Male, 8–14 week-old ApoE k/o mice and their corresponding control C57BL/6J mice were used. Significant reduction of cardiac histamine from 396.7±45.6 to 214.6±41.5 ng/g was observed over 120 min restraint stress with a corresponding increase in serum histamine from 126.9±4.0 to 188.4±17.3 ng/ml in C57BL mice. Cardiac mast cell activation was observed by light and electron microscopy. Both basal cardiac and serum histamine in ApoE k/o mice was significantly higher than that in C57BL mice. Although the extent of mast cell activation in ApoE k/o mice was similar to that of C57BL mice, the number of cardiac mast cells in ApoE k/o mice was 37% higher. Histamine levels were hardly detectable with or without stress in W/W* mast cell deficient mice.

Conclusions: Acute restraint stress triggered cardiac histamine release in mice that was clearly derived from mast cells, as it was absent in W/W* mice. The high basal cardiac and serum histamine in ApoE k/o mice, along with the high number of cardiac mast cells, suggest possible ongoing cardiac mast cell activation that may participate in atherosclerosis. These results may possibly help better understand stress-related cardiovascular pathology.

Keywords: Atherosclerosis; Coronary disease; Cytokines; Ischemia; Vasoactive agents

1. Introduction

Increasing evidence implicates acute psychological stress in cardiovascular pathology, especially silent myocardial ischemia (MI). MI occurring without angina on presentation now appears to be a sizable portion of the MI population [1–4]. Stress also precipitates or exacerbates certain neuroinflammatory conditions, many of which involve mast cells [5,6]. Mast cells are critical for allergic reactions, but they also release numerous vasoactive, neurosensitizing and inflammatory molecules [7]. Mast cells are located close to neurons (for review see Ref. [8]) where they can be activated by neuropeptides [9], by antidromic trigeminal ganglion stimulation [10], as well as by restraint stress [11,12]. These findings point to the significance of mast cell–neuron interactions [5] and have heightened interest in the versatile role of mast cells [13].

There is growing evidence that cardiac mast cells may participate in the development of atherosclerosis, coronary inflammation and cardiac ischemia [14–17]. Mast cells are increased and/or activated in association with MI [15] and atherosclerosis [16], as well as ischemic cardiomyopathy.
Mast cells were also recently shown to accumulate in the shoulder region of human coronary atheromas, the predilection sites of atheromatous erosion/rupture [15,16], and have also been implicated in coronary arteries during spasm or plaque rupture [14]. The human mast cell proteolytic enzyme chymase has been shown to be the main cardiac source of converting enzyme generating the coronary constrictor angiotensin II [19]. Cardiac mast cell derived histamine [20] can constrict the coronaries [21] and can sensitize nerve endings [22]; such an action is supported by recent findings showing adventitial mast cells localized close to nerve endings in atherosclerotic coronary arteries [23].

Acute restraint stress in rodents has been established as a useful model to study stress-related events especially those involving inflammatory processes [24]. It was shown that acute stress induced rat cardiac mast cells activation documented morphologically, an effect blocked by the ‘mast cell stabilizer’ disodium cromoglycate (cromolyn) [12]. However, the source of any mediators released was not investigated, neither was the possible contribution of this effect to atherosclerosis.

Here, we report that acute stress by restraint triggered cardiac histamine release, as evidenced by reduced cardiac histamine content and corresponding increases in serum histamine levels in C57BL and ApoE k/o mice. Although the extent of cardiac mast cell activation in ApoE k/o mice that develop atherosclerosis was similar to that of C57BL mice, the number of cardiac mast cells in ApoE k/o mice was higher than that in C57BL mice; moreover, both cardiac and serum histamine levels were significantly higher in ApoE k/o mice than in C57BL mice, indicating higher basal release of histamine possibly from on-going cardiac mast cell activation in ApoE k/o mice. Both cardiac and serum histamine levels in W/W<sup>+</sup> mice were very low and did not change with stress, suggesting that stress-induced cardiac histamine release was derived from mast cells.

2. Methods

2.1. Restraint stress

Normal (C57BL/6J), W/W<sup>+</sup> (WBB6F1/J-W/W<sup>+</sup>) genetically mast cell deficient mice and their wild type +/+ control (WBB6F1) mice, as well as ApoE k/o mice (JR2052 C57BL/6J-Apoe<sup>tm1 unc</sup>) for which C57BL/6J mice are considered their wild type controls (Jackson Laboratories, Bar Harbor, ME) were housed in plastic cages (four mice per cage) with a wire top in a modern animal facility under the supervision of veterinarians. Mice were allowed food and water ad libitum and were maintained in an automatic 14:10 h dark–light cycle. Animals were kept in the animal facility for at least 1 week before use. Each mouse was brought into an isolated procedure room, adjacent to the animal holding room inside the animal facility. The heart was cut into small pieces (about 1 mm³) and washed with phosphate buffered saline (PBS, Sigma, St. Louis, MO). Heart tissue fragments were then disrupted using a Polytron (Brinkmann Instruments, Westbury, NY) at 4 °C for 15 min and the supernatant was removed, immediately frozen on dry ice and kept at −80 °C until the histamine assay. The heart was cut into small pieces (about 1 mm³) and washed with phosphate buffered saline (PBS, Sigma, St. Louis, MO). Heart tissue fragments were then disrupted using a Polytron (Brinkmann Instruments, Westbury, NY) at 4 °C in PBS. The weight of tissue per total volume was recorded for each sample. The homogenized samples were centrifuged (15 000×g) at 4 °C for 15 min and the supernatant was collected. Histamine in both the heart and serum samples was measured by radioimmunoassay (<sup>125</sup>I-RIA kit, Immunotech, Westbrook, ME). For serum histamine levels, blood was collected from the neck vessels after decapitation and there was no significant difference in histamine levels between serum and plasma samples. Thereafter, only serum was used for all the assays. Therefore, blood samples were allowed to clot overnight at 2–8 °C before
centrifuging for 20 min at 2000 × g at 4 °C; the serum was then collected and kept at −80 °C until use.

2.4. Light microscopy

For light and electron microscopy, the heart was rapidly removed and fixed en bloc by immersion in 4% paraformaldehyde for 2 h at room temperature and then overnight at 4 °C. The tissue was frozen using Tissue Freezing Medium (Triangular Biomedical Sciences, Durham, NC) and thin sections (7 µm) were cut using a cryostat (Jung CM 3000, Leica, Inc. Deerfield, IL). Cardiac sections were stained with acidified (pH<2.5) toluidine blue (Sigma, St. Louis). Mast cells were counted at 400× in an area of 0.2948 mm² using six random cardiac sections from each mouse by two researchers blinded to the experimental conditions using a Diaphot inverted Nikon microscope (Don Santo, Natick, MA).

Results are presented as scattergram of percent activated mast cells stained with toluidine blue (the number of mice is shown in parentheses for each condition). Mast cell activation was defined as the presence of extruded granules close to the surface of the cell in question or staining of about half or less of the cell section with toluidine blue. A scattergram is used to appreciate the individual variation, while the mean±standard deviation is also provided for easier comparison. This subjective evaluation clearly records only mast cells that have been maximally stimulated.

2.5. Electron microscopy

Tissue samples were fixed in modified Karnovsky’s medium containing 2% paraformaldehyde, 3% glutaraldehyde and 0.5% tannic acid in 0.1 M cacodylate buffer (pH 7.4) and were processed as previously described [25]. They were examined and photographed using a Phillips-300 transmission electron microscope.

2.6. Statistical analysis

For histamine measurements, Student’s t-test was used to compare the differences between the stressed group and the control group. For light and electron microscopic observations, results were compared by non-parametric analysis using the Mann–Whitney U-test. Comparisons were done between C57BL control and stressed animals, while the W/W mast cell deficient mice were compared to their respective wild-type age-matched controls after both were stressed. The means and standard deviations of the results are reported in the text only, while the mean is also shown by a horizontal line in the scattergram. For corticosterone analysis, the P-values were obtained using a one-tailed t-test; for all of the other analyses, P-values were obtained using a two-tailed t-test. A P-value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Effect of acute stress on serum corticosterone levels

Serum corticosterone levels increased during stress (Fig. 1A–C). Acute restraint stress for 15 min rapidly raised these levels from 30.3±14.8 to 311.1±69.6 ng/ml (10-fold) in C57BL mice (Fig. 1A, *P<0.05), from 24.5±5.9 to 243.4±47.6 ng/ml (10-fold) in W/W mast cell deficient mice (Fig. 1B, *P<0.05) and from 42.7±26.4 to 338.6±114.7 ng/ml (8-fold) in ApoE k/o mice (Fig. 1C, *P<0.05). By 120 min, serum corticosterone levels were

![Fig. 1. Serum corticosterone levels were measured in control (unstressed) and stressed (A) C57BL (n=14); (B) W/W′ mast cell deficient (n=8); and (C) ApoE k/o mice (n=8). The mean±S.D. is derived from one measurement from each animal, the number of which is shown in the parentheses.](image-url)
3.2. Effect of acute stress on cardiac histamine levels

Histamine was measured in whole heart from control and stressed animals. Histamine levels in the hearts from control C57BL mice (n=14) were 354.3±160.1 and 445.0±196.8 ng/g tissue; these values decreased significantly to 211.1±97.2 ng/g (P<0.05) and 175.0±81.7 ng/g (P<0.05) after 15 and 30 min of stress, respectively. The reduction from 390.9±130.2 to 257.8±151.8 ng/g after 2 h of stress was not significant (P>0.05) (Fig. 2A). This result confirms the morphological findings of mast cell activation (see below) and shows that at least one mediator is in fact secreted from cardiac mast cells in response to acute stress.

W/W⁻ mast cell deficient mice (n=8) had very low histamine levels in the heart either in control mice (25.8±9.0, 22.5±7.1 and 25.0±9.3 ng/g) or after they were stressed (22.6±10.3, 23.4±7.1 and 25.0±12.0 ng/g) (Fig. 2B). In contrast, their wild type +/+ control mice (n=8) showed similar results to C57BL mice. The unstressed +/+ mice had 422.5±211.9 and 422.5±245.3 ng/g of cardiac histamine, which after 15 and 30 min of stress significantly decreased to 197.5±66.1 ng/g (P<0.05) and 176.3±86.8 ng/g (P<0.05), respectively. The reduction after 2 h of stress from 410.0±185.4 to 213.5±112.33 ng/g was not significant (P>0.05) (Fig. 2C). These results imply that nearly all of the histamine secreted under stress comes from mast cells.

The activation of cardiac mast cells in ApoE k/o mice was also significantly induced by restraint stress. Cardiac histamine levels in ApoE k/o mice (n=8) were 520.0±206.2 and 481.3±214.5 ng/g; these values decreased significantly after 15 and 30 min of stress to 310.0±99.0 ng/g (P<0.05) and 296.7±165.6 ng/g (P<0.05), respectively. The reduction after 2 h of stress from 511.3±231.6 to 418.33±107.41 ng/g was not significant (P>0.05) (Fig. 2D). The mean basal cardiac histamine level of 504.2±20.3 ng/g in ApoE k/o mice was significantly higher than that of 396.7±45.6 ng/g in C57BL mice (P<0.05), and so was the level after stress, which was 341.7±66.7 ng/g in ApoE k/o mice and 214.6±41.5 ng/g in C57BL mice (P<0.05) (Fig. 2E). These results indicate that ApoE k/o mice may have increased number of mast cells that appear to be already activated before stress in association with atherosclerosis.

3.3. Effect of acute stress on serum histamine levels

Acute stress significantly increased serum histamine levels (Fig. 3A) in C57BL mice from a basal level of 124.2±17.9, 125.0±17.5 and 131.5±32.3 ng/ml at 15 min, 30 min and 2 h to 193.4±48.4 (P<0.05), 169.1±33.8 (P<0.05) and 202.5±27.5 ng/ml (P<0.05), respectively after stress.

W/W⁻ mast cell deficient mice showed almost undetectable serum histamine levels that did not change with stress (Fig. 3B), whereas +/+ mice showed increased serum histamine levels similar to those of C57BL mice after different periods of stress (Fig. 3C). Therefore, any histamine increased in the serum of the +/+ controls, could not have derived from any other source than mast cells.

Serum histamine in ApoE k/o mice was also increased by stress over different periods of time (Fig. 3D). Moreover, the average serum histamine in control unstressed ApoE k/o mice was significantly higher (161.1±4.0 ng/ml) than that of C57BL mice (126.9±4.0 ng/ml) (P<0.05) (Fig. 3E) from which they derive, further indicating increased basal histamine secretion in these mice. Overall, the average (results from all time points combined) serum histamine level of 188.4±17.3 ng/ml in stressed C57BL mice was significantly higher than that of 126.9±4.0 ng/ml in unstressed ones (P<0.05) (Fig. 3E). Whereas ApoE k/o mice already show significantly higher serum histamine at rest (161.1±4.0 ng/ml) than C57BL mice (P<0.05) (Fig. 3E), the average serum histamine level after stress (201.7±19.9 ng/ml), although significantly higher than its own controls (Fig. 3E), was similar to that of C57BL mice (188.4±17.3 ng/ml) (P>0.05).

3.4. Effect of acute stress on cardiac mast cell activation

Most cardiac mast cells were located close to blood vessels (Fig. 4A). Mast cells in control C57BL mice showed minimal activation as evidenced by light microscopy (Fig. 4A). Instead, those from stressed mice were activated to varying degrees, ranging from involvement of part of the cell (Fig. 4B,C) to almost complete loss of staining, indicating maximal activation (Fig. 4D). Ultrastructural studies showed that mast cells from control animals contained mostly intact, round, homogeneous electron dense secretory granules (Fig. 5A). In contrast, mast cells from stressed animals were activated to various degrees ranging from mild secretory granule changes (Fig. 5B), to extensive granule swelling (Fig. 6A,B). Some mast cells showed complete dissolution of the electron dense secretory granule content, although frank exocytosis was not apparent (Fig. 6C). Some of the milder changes observed with electron microscopy or those mast cells showing complete loss of staining (e.g. mast cell in Fig. 4D) may not be easily apparent at the light microscopic
The extent of mast cell activation was estimated by averaging the counts taken at the light microscope by two different investigators. Acute restraint stress for 30 min clearly increased the extent of mast cell activation in the heart in C57BL mice (Fig. 7) from 25.6±1.8 to 40.9±2.2% (P<0.05). The extent of activation was also assessed in ApoE k/o animals that develop coronary atherosclerosis. Mast cell activation in the heart of un-stressed ApoE k/o mice was 26.7±4.5%, while 30 min stress increased it to 37.9±7.3% (P<0.05), this degree of activation was similar to that of C57BL mice. However, the number of cardiac mast cells counted from six random cardiac sections of ApoE k/o mice was 37% higher than that of C57BL mice. These findings suggest that some of...
Fig. 3. Serum histamine levels were measured in control (unstressed) and stressed (A) C57BL (n=14); (B) W/W$^+$ mast cell deficient mice (n=8); (C) $+/+$ mice (n=8); (D) ApoE $k/o$ mice (n=8); and (E) composite comparison of the mean serum histamine levels from all time points of control and stressed mice. The mean±S.D. is derived from one measurement from each animal, the number of which is shown in the parentheses.

The cardiac mast cells in ApoE $k/o$ mice may already be maximally activated and not recognizable by light microscopy.

We also investigated the effect of stress on the wild type $+/+$ mice of the W/W$^+$ genetically mast cell deficient mice. Control, unstressed $+/+$ animals showed 27.4±4.1% mast cell activation in the heart which was increased to 46.7±9.7% ($P<0.05$) by 30 min stress (Fig. 7). Meanwhile, the number of cardiac mast cells counted from cardiac sections of $+/+$ mice was similar to that
Fig. 4. Light photomicrographs of heart sections from C57BL mice stained with toluidine blue to show cardiac mast cells (A) in control (unstressed) (B, C and D) stressed mice. Empty granules stain pink (arrowhead) as compared to the dark blue color of intact granules (solid arrows); note almost totally activated mast cell in (D). Bar=10 μm.

from C57BL mice. The W/W° mast cell deficient mice had no detectable mast cells in the heart to estimate mast cell activation either before or after stress.

4. Discussion

The present study demonstrated that acute restraint stress triggered mouse cardiac histamine release evidenced by decrease in heart histamine by almost 46% and increase in serum histamine by about 48%. Mast cell activation was also documented by light and electron microscopy. Acute restraint stress in rodents is widely used to study stress-related events and was also shown to activate mast cells in the skin [24] and the dura [11]. The stress response, as indicated by the serum levels of corticosterone, was equivalent in the C57BL, W/W° and ApoE k/o mice that develop atherosclerosis [26]. Consequently, any observed difference in histamine release and mast cell activation could not be due to any difference in the activation of the hypothalamic–pituitary–adrenal axis. Cardiac mast cell activation was present in control unstressed mice (25.6% activated, 126.9 ng/ml serum histamine). This could be a functional response to the stress of handling the mice even though experiments were carried out inside the animal facility that had been shown to reduce basal activity from the higher levels seen in the laboratory [12]. Histamine measured by RIA includes both bound and unbound amine in the heart tissue. The serum histamine is mostly unbound as measurements in plasma did not differ from those in serum. Blood histamine could, theoretically, come from any tissue containing mast cells. Our results showed that significant increase of serum histamine was evident at 15 min, a time possibly too short for histamine from other sources to reach the systemic circulation. Evidence that mast cell derived histamine from other tissues, such as the skin, is not likely to contribute to blood levels significantly comes from a report showing that serum histamine levels are not high in systemic mastocytosis patients [27]. The heart histamine levels at 120 min were not significantly different from controls. This finding may be due to a
feedback inhibition of serum histamine on further cardiac histamine release through the activation of auto-inhibitory H-3 receptors [28]; alternatively, or additionally, cardiac histamine stores may be replenished over time.

Here, we included ApoE k/o mice in order to investigate whether acute stress would have any different effect on cardiac histamine release and serum histamine levels in these mice, since mast cells have been reported to be associated with atherosclerosis and MI [17]. Our results showed that both the basal cardiac and serum histamine levels were significantly higher in ApoE k/o mice than those in C57BL mice. Although the extent of cardiac mast cell activation before and after stress was similar between C57BL mice and ApoE k/o mice, the number of cardiac mast cells counted in ApoE k/o mice was higher than that from C57BL mice. We believe that these increases in histamine levels and number of cardiac mast cells may be mostly due to mast cell recruitment in relation to mast cell growth factor C-kit ligand (stem cell factor) that was increased in association with coronary mastocytosis in reperfusion ischemia [29]. Alternatively, RANTES (regulated upon activation, normal T cell expressed and presumably secreted) may be involved as it has been shown to be particularly chemotactic for mast cells [30]. These findings suggest that an increased number of activated mast cells in the heart and coronary arteries in ApoE k/o mice may be involved in the pathology of coronary atherosclerosis. Stress-induced cardiac histamine release may help explain stress-related myocardial ischemia that could progress to MI when the underlying coronary pathology is more severe; this may have been true in the case of double k/o (ApoE and low-density lipoprotein

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**Fig. 5.** Transmission electron micrographs of cardiac mast cells from (A) control, unstressed and (B) stressed C57BL mice showing numerous secretory granules that have released their contents (arrowheads); note tissue from both control and stressed mice have numerous intact granules (solid arrow). Bar=1 \( \mu \)m.
Histamine actually provoked acute coronary spasm in patients with non-exertional chest pain [36] and plasma histamine elevations correlated with the onset of acute MI in patients [37]. Anaphylaxis has also been associated with increased incidence of MI in two retrospective population studies [32,38]. Human mast cells also secrete chymase, which can generate the vasoconstrictive angiotensin II [19,39,40], as well as histamine-releasing peptides [41], thus propagating mast cell activation. Histamine would also sensitize local neurons [22], potentially generating a pro-arrhythmogenic effect since mast cells are found close to the sinoatrial node [42], and have been shown to have functional associations with nerve endings [43]. In fact, adventitial mast cells in atherosclerotic coronary arteries were recently found localized close to sensory nerve endings [23]. Mast cells could serve as a link between the immune and nervous systems [5,44] since neuropeptides receptor deficient) mice, in which acute stress induced MI [31].

Histamine released primarily from the heart [20,32] during the early phase of acute stress could influence coronary artery disease by: (a) causing direct constriction [21]; (b) potentiating other constrictors in atherosclerotic coronary arteries [33]; (c) increasing the thickness of the intima [34]; and (d) inducing pro-inflammatory cytokine production from human coronary endothelial cells [35].
addition to histamine. Our preliminary results actually showed that acute restraint stress induced serum increase in IL-6 that was 4-fold higher in ApoE k/o than in C57BL mice after 120 min of restraint stress [50]. Activated mast cells may also be involved in atherosclerotic process since previous studies showed that mast cell granules were associated with uptake of low-density lipoprotein by macrophages [51] that leads to the formation of foam cells—a characteristic pathological change in early atherosclerosis.

Mast cells are known to be present in the heart and have been shown to have different characteristics from those in the skin [18]. The possible role of mast cells in coronary artery disease (CAD) was reviewed recently [52]. The notion that mast cells may be involved in the pathophysiology of atherosclerosis [15] is supported by their increased presence [16,53] and/or activation in atheromatous plaques [54], especially in relation to coronary spasm [14] and coronary plaque rupture [55]. We showed that ApoE k/o had increased number of cardiac mast cells, many of which were degranulated under stress in the vicinity of lipid congested coronary arteries. Increased numbers of degranulated mast cells were also observed in the adventitia of ruptured atherosclerotic coronary artery plaques in association to MI in humans [17]. Our ultrastructural observations indicated the presence of maximally activated mast cells that would be missed by light microscopy. Such mast cells have previously been termed ‘phantom’ mast cells [56] and were described in scleroderma [56], fatal cases of which have been associated with myocardial mast cell infiltration [57]. Moreover, cardiac mast cells were reported to be increased in ischemic cardiomyopathy [58] and in reperfusion ischemia [29].

Mast cell–neuron interactions [8] that are increasingly implicated in neuroinflammatory disorders [5], have led to the conclusion that mast cells have a more versatile role [13]. Acute stress can worsen or precipitate [1–4] cardiac ischemia especially in patients with CAD [4,59]. The present findings suggest that cardiac mast cell activation by acute stress may contribute to this pathology through the release of histamine or proinflammatory mediators.

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