Stress-induced interleukin-6 release in mice is mast cell-dependent and more pronounced in Apolipoprotein E knockout mice

Man Huang[a], Xinzhu Pang[b], Katia Karalis[c], Theoharis C. Theoharides[a],*

[a]*Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA*
[b]*Department of Pathology, Tufts University School of Medicine, New England Medical Center, Boston, MA 02111, USA*
[c]*Division of Endocrinology, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA*

Abstract

Objective: Interleukin-6 (IL-6) is elevated in the serum of patients with coronary artery disease (CAD) and acute coronary syndromes (ACS). Intracoronary release of IL-6 was reported in ACS that can also be triggered by acute stress. In rats, acute restraint stress increases serum IL-6 and histamine, both of which may derive from mast cells. The current study was carried out in order to determine any possible difference in stress-induced IL-6 release in atherosclerotic mice and the contribution of mast cells, corticotropin-releasing hormone (CRH) and urocortin (Ucn). **Methods:** C57BL/6J, W/W^v mast cell-deficient, Apolipoprotein E (ApoE) and CRH knockout (k/o) mice were stressed in plexiglass restraint chambers for 15 to 120 min. Serum corticosterone and IL-6 levels were measured. Some C57BL and ApoE k/o mice were pretreated with either the mast cell stabilizer disodium cromoglycate (cromolyn) or the peptide CRH receptor (CRH-R) antagonist Astressin. Cardiac mast cell activation was studied in both C57BL and ApoE k/o mice using light microscopy. **Results:** Acute stress increased serum IL-6 in mice, an effect much greater in ApoE k/o atherosclerotic mice. Stress-induced IL-6 release was absent in W/W^v mast cell-deficient mice and it was partially inhibited by cromolyn in C57BL and ApoE mice. Mast cells were found adjacent to atherosclerotic vessels in ApoE k/o mice and were activated after stress. CRH k/o mice released more IL-6 in response to stress than their wild types, but Astressin significantly inhibited IL-6 release. Stress-induced IL-6 release may, therefore, be at least partly due to peripheral Ucn and/or CRH, with Ucn possibly overcompensation for CRH deficiency. **Conclusion:** The present findings indicate that acute stress leads to mast cell-dependent serum IL-6 increase that may help explain stress-related coronary inflammation.
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Keywords: Atherosclerosis; Coronary disease; Cytokines; Inflammation; Stress

1. Introduction

Acute psychological stress is implicated in a variety of neuroendocrine–immune interactions and numerous neuro-inflammatory syndromes [1]. It is also a known risk factor for coronary artery disease (CAD), especially silent myocardial ischemia [2–6]. Acute stress activates the hypothalamic–pituitary–adrenal (HPA) axis through the release of corticotropin-releasing hormone (CRH), which regulates the stress response [1]. Hypothalamic CRH, theoretically, is anti-inflammatory because of the systemic release of glucocorticoids. However, unlike its central effect, peripherally synthesized CRH has a proinflammatory effect [1,7], some of which may be contributed by the CRH-related peptide urocortin (Ucn) [8,9].

Acute psychological stress can trigger the onset of acute coronary syndromes (ACS) [10] in patients with CAD, in which the proinflammatory cytokine interleukin-6 (IL-6) [11] is elevated [12,13]. Acute stress also causes plasma IL-6 elevation in rodents [14,15] and precipitates myocardial infarction (MI) in atherosclerotic mice deficient both in apolipoprotein E (ApoE) and in low-density lipoprotein receptor (LDL-R) [16], but not in normal mice. However, the possible reason for the different impact of stress on

*Corresponding author. Tel.: +1-617-636-6866; fax: +1-617-636-2456.

E-mail address: theoharis.theoharides@tufts.edu (T.C. Theoharides).

normal and atherosclerotic mice and the mechanism of stress-induced IL-6 release remain largely unknown.

IL-6 was originally identified in monocytes/macrophages, fibroblasts and endothelial cells [11]. It is a pleiotropic cytokine that has multiple functions, including: (a) stimulatory effects on the proliferation and differentiation of lymphocytes [11]; (b) activation of the HPA axis independent of CRH; (c) induction of the hepatic acute phase response proteins, particularly C reactive protein (CRP), with both IL-6 and CRP being strong predictors of cardiovascular mortality; and (d) increase of fatty lesions and deterioration of early atherosclerosis in ApoE knockout (k/o) mice. The importance of IL-6 in the pathophysiology of CAD has been documented in several studies [17,18] and it is probably the link between inflammation, stress and CAD [19].

Mast cells are well known for their involvement in allergic reactions [20] and neuroinflammatory conditions that are precipitated or exacerbated by stress [21]. Mast cells are not only a rich source of the vasoactive and pro-arrhythmogenic molecule histamine, but also abundant in cytokines, including IL-6 [22]. Increased numbers of activated cardiac mast cells are found in ventricles, the sinoatrial node and the shoulder region of the fibrous plaque in association with atherosclerosis [23,24]. It has also been reported that coronary inflammation may depend on mast cell-derived mediators [25]. We previously showed that acute stress activated cardiac mast cells in rats [26] and mice in which there was histamine release that was higher in ApoE k/o mice [27]. A recent editorial highlighted the importance of mast cell activation by stress and urged further studies that might explain the mechanism of such activation [28].

Here, we report that acute stress caused IL-6 release in mice, an effect greater in ApoE k/o atherosclerotic mice, at least partly due to peripheral CRH/Ucn since the peptide CRH receptor (CRH-R) antagonist Astressin significantly inhibited IL-6 release. The contribution of mast cells to stress-induced IL-6 release is remarkable since it is absent in W/W^v mast cell-deficient mice. Mast cells were found adjacent to atherosclerotic vessels in ApoE k/o mice and were activated after stress. These findings may help explain stress-related cardiovascular pathology, especially since intracoronary release of IL-6 was recently documented in ACS [12].

2. Methods

2.1. Restraint stress

Eight- to 20-week-old C57BL/6J mice ($n=14$), W/W^v mast cell-deficient mice (WBB6F1/J-W/W^v) ($n=8$) and ApoE (JR2052 C57BL/6J-Apoe^{tmlunc}) k/o mice ($n=8$) (Jackson Laboratories, Bar Harbor, ME, USA) for which C57BL mice are considered as their wild type controls, as

well as CRH k/o mice ($n=6$) [29] (Children's Hospital, Boston) and their respective controls were allowed food and water ad libitum and were maintained in a 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 inside the animal facility between 9 and 11 a.m. (to avoid any effect of diurnal rhythms) for 30 min every day for 3 days in order to reduce the stress of handling. During the day of the experiment, each control animal was allowed to stay in its cage for the designated period of time on a bench top at room temperature in the procedure room. At a different time, the experimental mouse was placed in a clear, plexiglass restraint chamber (Harvard Apparatus, Cambridge, MA, USA) for the designated times. No mouse was ever in close proximity while another was stressed or dissected. At the end of the experiment, each animal was killed by asphyxiation over CO_2 vapor and decapitated. Blood was collected for corticosterone and IL-6 measurements. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Light 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 (Triangle Biomedical Sciences, Durham, NC, USA) and thin sections (7 μm) were cut using a cryostat (Jung CM 3000, Leica, Deerfield, IL, USA). Cardiac sections were stained with: (a) 0.25% acidified (pH <2.5) toluidine blue (Sigma, St. Louis, MO, USA) for 15 min at room temperature to identify mast cells, (b) Hematoxylin–Eosin (Sigma) to demonstrate cardiac muscle fibers, or (c) saturated, freshly filtered Sudan black-diacetine (Sigma) aqueous (1:1) solution for 30 min at room temperature to stain the intracoronary fatty deposits. Mast cells were counted at 400× (an area of 0.0726 mm^2) in 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, USA). 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 [27].

2.3. Corticosterone measurements

Blood samples were allowed to clot overnight at 2–8 °C before centrifuging for 20 min at approximately 2000×g. The serum was collected and subjected to corticosterone radioimmunoassay using the Corticosterone ^{125}I-RIA kit (ICN, Costa Mesa, CA, USA).

2.4. IL-6 measurements

Mouse serum and plasma samples were both assayed for IL-6 with the quantitative sandwich enzyme immunoassay technique (Quantikine M Murine, Mouse IL-6, R&D Systems, Minneapolis, MN, USA) and no significant difference was found between the two; serum samples were used thereafter.

2.5. Drug pretreatment

To determine the contributions of peripheral CRH/Ucn and mast cells to stress-induced IL-6 release, some C57BL and ApoE k/o mice were pretreated with an intraperitoneal (i.p.) injection of either the non-selective peptide CRH-R antagonist Astressin (0.5 mg/kg dissolved in 0.1 M PBS, Neurocrine, La Jolla, CA, USA) 10 min prior to 120 min restraint stress ($n=8$), or the mast cell stabilizer disodium cromoglycate (cromolyn, 25 mg/kg in 0.1 M PBS, Sigma) 30 min prior to 120 min of restraint stress ($n=8$).

2.6. Statistical analysis

One-way ANOVA run by SigmaStat was used to compare the differences between the stressed and unstressed group, as well as among different treatment groups. Results are presented as means±standard deviation. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of acute stress on serum corticosterone levels

Serum corticosterone levels in all control animals were less than 100 ng/ml (Fig. 1A–F), but increased similarly by 120 min of stress in C57BL mice (456.6±66.8 ng/ml, Fig. 1A, $P<0.05$), ApoE k/o mice (453.3±66.6 ng/ml, Fig. 1B, $P<0.05$) and in W/W^v mast cell-deficient mice (389.9±144.4 ng/ml, Fig. 1C, $P<0.05$). Pretreatment with the peptide CRH-R antagonist Astressin (0.5 mg/kg, i.p.) for 10 min decreased serum corticosterone level by 40.9% to 282.0±63.7 ng/ml ($n=8$) (Fig. 1D, $P<0.05$). Pretreatment with the mast cell stabilizer cromolyn (25 mg/kg, i.p.) did not have any effect on serum corticosterone levels ($n=8$) (Fig. 1E, $P>0.05$). As expected, there was no increase in serum corticosterone in CRH k/o mice ($n=6$) after stress (Fig. 1F, $P>0.05$).

3.2. Effect of acute stress on IL-6 release in normal C57BL and ApoE k/o mice

Acute stress increased IL-6 release in C57BL mice in a time-dependent fashion from less than 15 pg/ml to 34.1±15.2 pg/ml ($P=0.057$) after 60 min, and 50.8±5.6 pg/ml ($P<0.05$) after 120 min (Fig. 2A).

The basal IL-6 levels in the serum of ApoE k/o mice were similar to those in C57BL mice. However, the serum level of 172.9±84.2 pg/ml at 120 min of stress was three-fold higher than that released from C57BL (Fig. 2A and B, $P<0.05$).

3.3. Effect of acute stress on cardiac mast cells in normal C57BL and ApoE k/o mice

Cardiac mast cells were easily identified after staining with toluidine blue; in control mice mast cells were mostly perivascular, but appeared intact since they were uniformly stained blue (Fig. 3A). It should be noted that metachromasia is often not apparent if the section is cut so that multiple layers of granules are stacked or if the section is thick or overstained. In contrast, mast cells from mice stressed for 30 min showed obvious signs of activation with partial loss of their granular staining and evidence of metachromatic granule content outside the cells (Fig. 3B). Staining with Hematoxylin/Eosin and counterstaining with toluidine blue permitted visualization of mast cells between cardiac muscle fibers (Fig. 3C and D), in which mast cell activation was apparent after 30 min of stress (Fig. 3C and D, note arrowheads). Coronary arteries from ApoE k/o mice were often found to be congested with platelets and erythrocytes (Fig. 4A) with mast cells localized in close proximity as shown in one representative section from an unstressed mouse (Fig. 4A). Staining for fatty deposits with Sudan black showed that coronary arteries in ApoE k/o mice also had substantial fatty deposits, stained grey-black; one such section shows a coronary artery from a stressed ApoE k/o mouse partially filled with fatty deposition and with one mast cell showing signs of activation (Fig. 4B). Quantitation of cardiac mast cells indicated that the total number of mast cells was 217±13 in control C57BL mice ($n=11$) and 221±17 after 120 min of restraint stress (Table 1). However, the corresponding number of cardiac mast cells in ApoE k/o mice ($n=9$) was substantially higher at 480±15 and 504±19, respectively ($P<0.05$, Table 1). The number of perivascular mast cells was also increased from 59.3±8.2 and 63.5±11.4% in control and stressed C57BL mice to 79.6±12.4 and 88.7±9.5% in ApoE k/o mice, respectively ($n=11$, $P>0.05$, Table 1). The extent of mast cell activation due to 120 min of restraint stress was about the same in the two groups and increased from 25.4±1.5 to 37.4±1.7% in C57BL mice ($n=11$) and from 26.7±4.5 to 46.4±3.9% ($n=9$, $P<0.05$ in each case, Table 1).

3.4. Involvement of mast cells in stress-induced IL-6 release

The average basal serum IL-6 levels in W/W^v mice were similar to those in C57BL mice (Fig. 2A and 5B). The surprising finding was that, after 60 min of restraint, serum IL-6 in W/W^v mice was only 7.3±2.9 pg/ml as
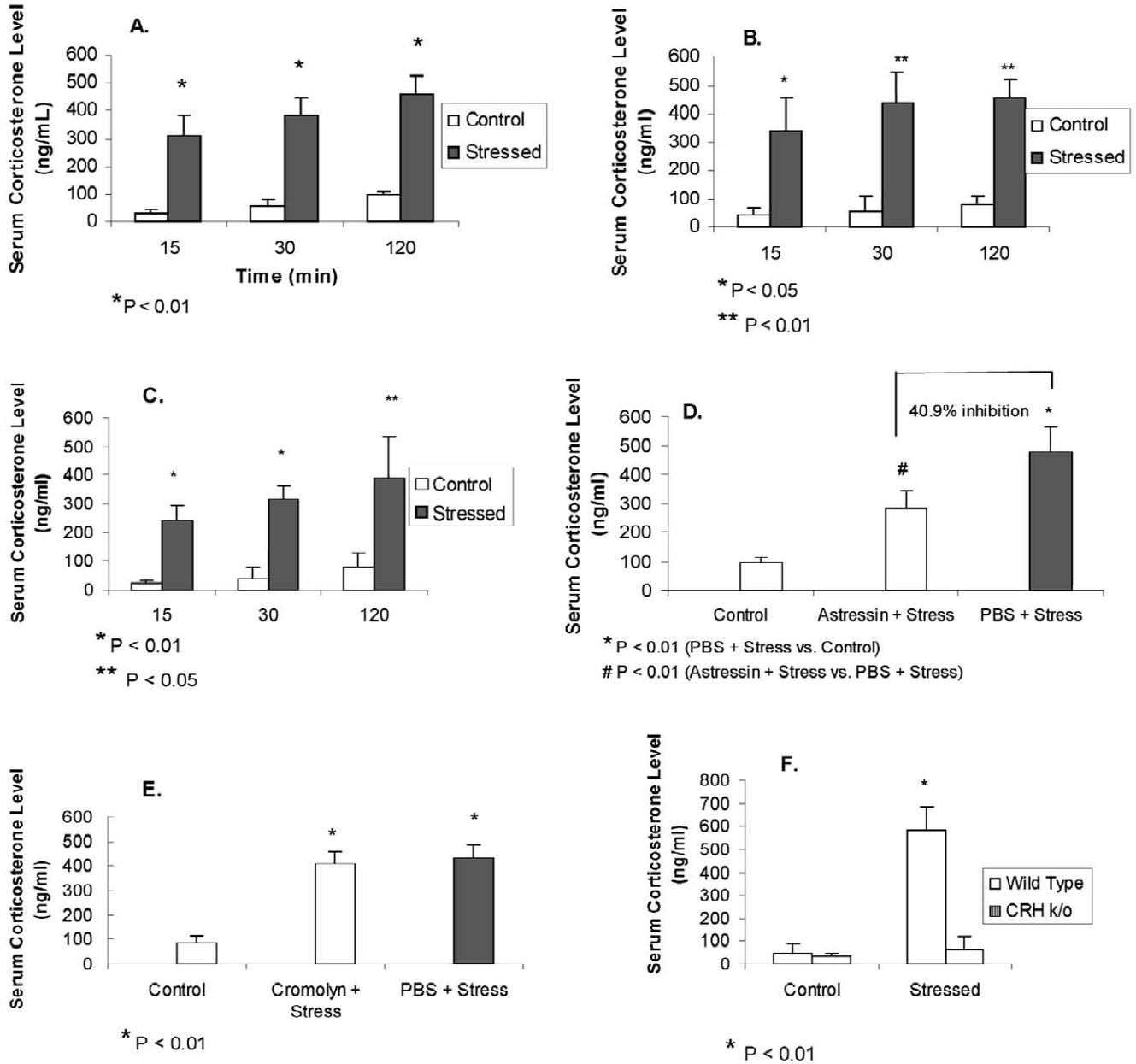
Fig. 1. Comparison of serum corticosterone levels in (A) C57BL/6J ($n=14$); (B) ApoE k/o ($n=8$); (C) W/W^v mast cell-deficient ($n=8$); (D) C57BL mice pretreated with the CRH-R antagonist Astressin ($n=8$) or PBS ($n=8$) before 2 h of stress; (E) C57BL mice pretreated with the mast cell stabilizer cromolyn ($n=8$) or PBS ($n=8$) before 2 h of stress; and (F) CRH k/o ($n=6$) and their wild type mice ($n=6$). (A–C) Based on the same data used to generate Fig. 1A–C of Ref. [27]. We included Fig. 1A–C in our current manuscript for the convenience of the readers to compare the serum corticosterone levels in stressed and unstressed mice among different strains.

compared to 34.1 ± 15.2 pg/ml in C57BL mice (Fig. 2A and 5B, $P<0.05$). When the duration of restraint stress was extended to 2 h, serum IL-6 levels in W/W^v mice still remained at a very low level of 10.5 ± 7.2 pg/ml (Fig. 5B).

Pretreatment with cromolyn (25 mg/kg, i.p.) for 30 min prior to 120 min of stress inhibited IL-6 release by 29.5% in C57BL mice, but this effect was not statistically significant (Fig. 5A, $P>0.05$). IL-6 release was also partially inhibited by cromolyn administered similarly in ApoE k/o mice by 46.5% (Fig. 5C, $P>0.05$), but this

effect was also not statistically significant due to the large standard deviation.

3.5. Effect of CRH/Ucn on IL-6 release

Pretreatment with Astressin (0.5 mg/kg, i.p.) for 10 min prior to 120 min of stress in C57BL mice decreased IL-6 release by 58.5% (Fig. 6A, $P<0.05$). Although CRH k/o mice and their wild type controls showed equivalent basal serum IL-6 levels at rest (Fig. 6B), after 60 min of
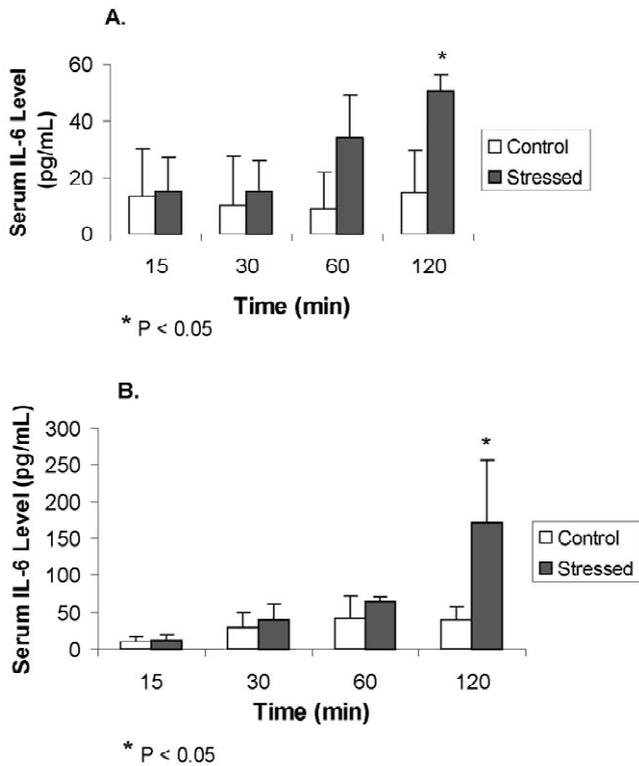
A.

B.

Fig. 2. Comparison of serum IL-6 levels in (A) C57BL/6J ($n=14$) and (B) ApoE k/o ($n=8$) mice.

restraint, serum IL-6 in CRH k/o mice was 34.5 ± 8.0 pg/ml versus 12.7 ± 2.9 pg/ml in the wild type mice (Fig. 6B, $P<0.05$). This higher serum IL-6 level in CRH k/o mice may be due to greater availability of Ucn, also shown to be more potent than CRH in stimulating IL-6 release, as a compensatory mechanism.

4. Discussion

In our current study, the response to acute restraint stress, as indicated by the serum levels of corticosterone, was equivalent among C57BL, ApoE k/o and W/W^v mast cell-deficient mice, as we reported previously [27]. Consequently, any observed difference in the serum IL-6 level could not be solely due to fluctuations in corticosterone levels. Our results show that ApoE k/o mice mounted a massive increase of serum IL-6 under stress, with a three-fold higher IL-6 level at 2 h than that of C57BL mice. This higher IL-6 release may contribute to the cardiac pathology of ApoE k/o mice [30] since IL-6 is expressed in atherosclerotic lesions of ApoE k/o mice [31] and exogenous IL-6 increases fatty lesions and exacerbates early atherosclerosis in these mice [32]. Moreover, acute stress was shown to precipitate MI in ApoE and low-density lipoprotein receptor (LDLR) double k/o mice [16]. The recent demonstration of cardiac release of IL-6 into the
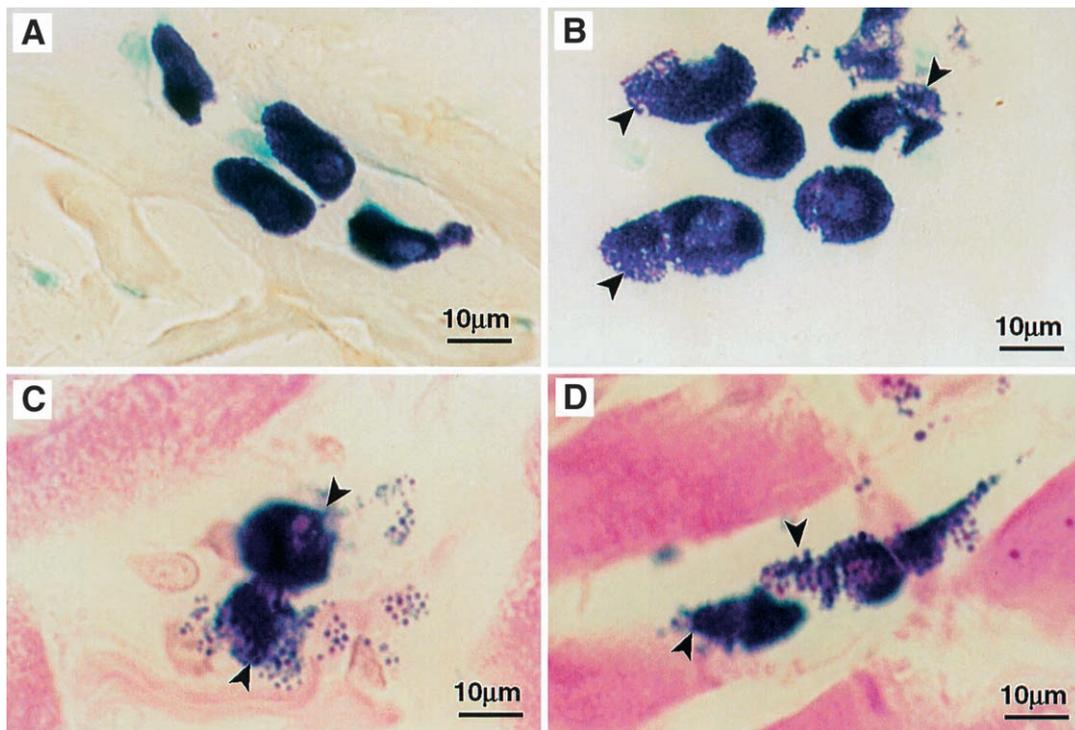

Fig. 3. Light photomicrographs of C57BL mouse heart mast cells from (A) control mouse and (B) stressed mouse stained with toluidine blue, as well as from (C, D) stressed mice stained with Hematoxylin–Eosin and counterstained with toluidine blue. Arrowhead identifies activated mast cells. Bar=10 μm.
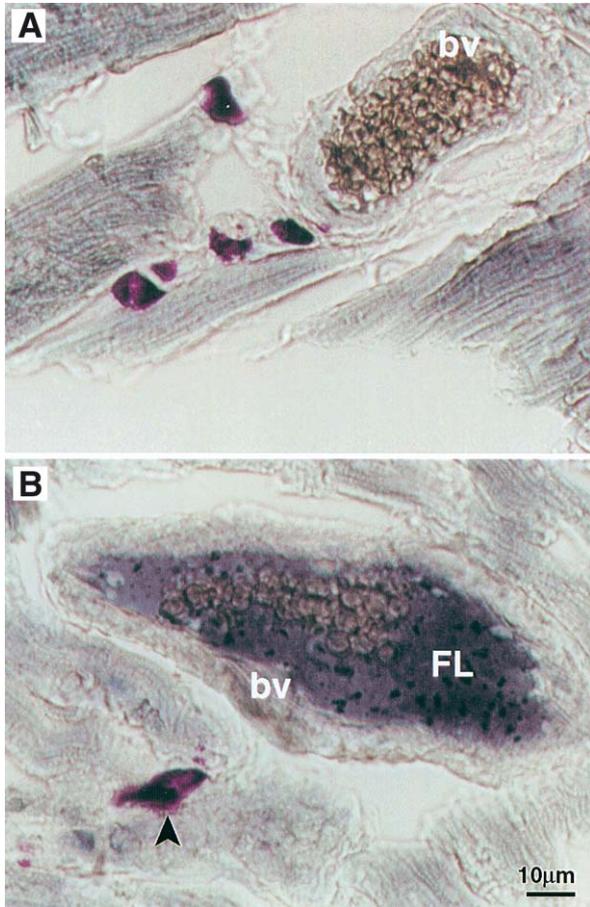
Fig. 4. Light photomicrographs of sections of coronary vessel from ApoE k/o mice stained with Sudan black to identify intraluminal fatty lesions (FL) and counter-stained with toluidine blue to identify mast cells. (A) A blood vessel from a control mouse congested with platelets and erythrocytes with apparently intact mast cells in the vicinity and (B) a blood vessel from an ApoE mouse stressed for 120 min containing platelets and a large fatty lesion (FL) occluding almost half of a blood vessel (bv) with activated mast cells nearby. Bar=10 μm.

coronary circulation in patients with ACS provides evidence of IL-6 release from a cardiac source in a clinical setting [12]. High cardiac IL-6 levels may contribute to local coronary inflammation, which plays a crucial role in

Fig. 5. Comparison of serum IL-6 levels in (A) C57BL mice pretreated with the mast cell stabilizer cromolyn ($n=8$) or PBS ($n=8$) before 2 h of stress; (B) W/W^v mast cell-deficient ($n=8$); and (C) ApoE k/o mice pretreated with the mast cell stabilizer cromolyn ($n=8$) or PBS ($n=8$) before 2 h of stress.

the pathophysiology of cardiovascular disease, especially CAD [33–35].

We previously showed that acute stress induces cardiac mast cell activation and histamine release, both of which were increased in ApoE k/o mice [27]. The number of mast cells counted from random cardiac sections of ApoE k/o mice was about 37% more than that in C57BL mice [27]. An accompanying editorial to this latter paper

Table 1
Number, proximity to atherosclerotic vessels and activation of cardiac mast cells (MC)

	Total No. MC	MC close to blood vessels (%)	Activated MC (%)
C57BL ($n = 11$)			
Control	217±13	59.3±8.2	25.4±1.5
Stressed	221±17	63.5±11.4	37.4±1.7*
ApoE knockout ($n = 9$)			
Control	480±13*	79.6±12.4[+]	26.7±4.5
Stressed	504±19*	88.7±9.5[+]	46.4±3.9*

*$P<0.05$ comparing 120 min restraint stress vs. control. [+]$P<0.05$ comparing the ApoE k/o to C57BL mice; all blood vessels contained erythrocytes and/or lipid material identified with Sudan black staining.
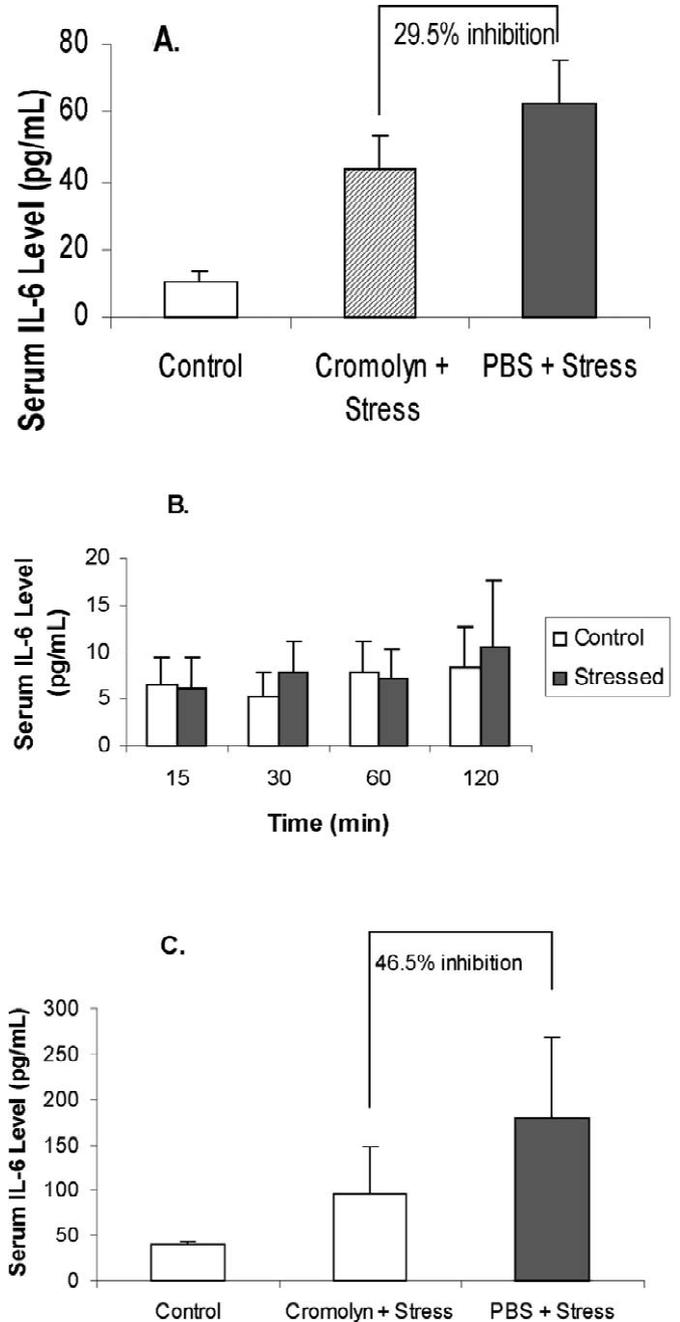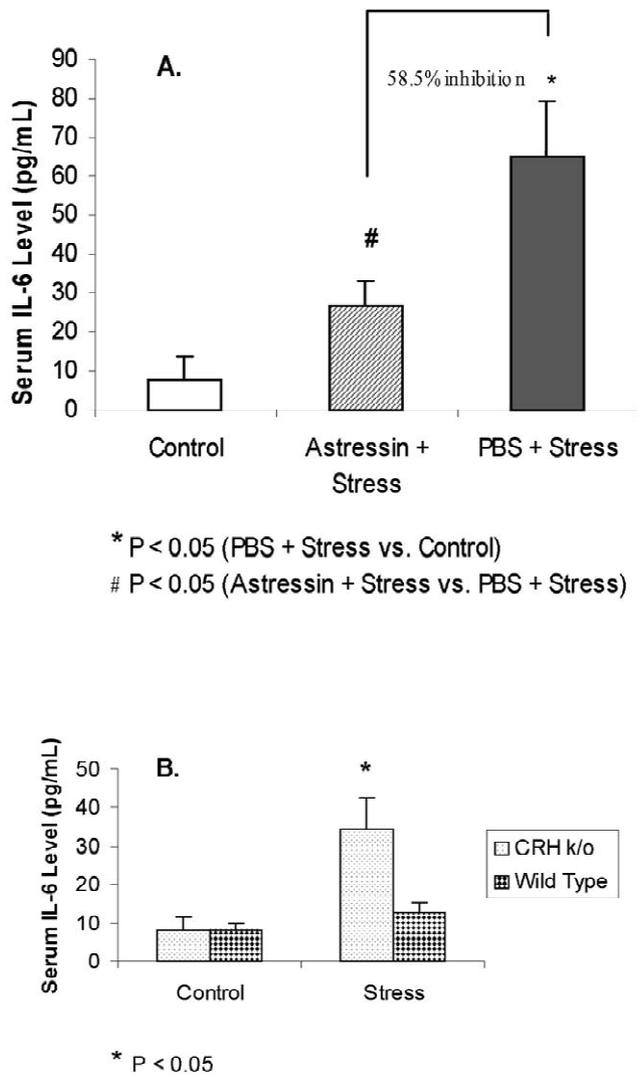
Fig. 6. Comparison of serum IL-6 levels in (A) C57BL mice pretreated with the CRH-R antagonist Astressin ($n=8$) or PBS ($n=8$) before 2 h of stress; and (B) CRH k/o ($n=6$) and their wild type control mice ($n=6$).

stressed the importance of understanding the mechanisms responsible for cardiac mast cell activation and their contribution to the onset and progression of atherosclerotic disease [28]. Our present data show that stress-induced IL-6 release is absent in W/W^v mice after restraint stress, indicating that this process is mast cell-dependent. In other studies using W/W^v mast cell-deficient mice, their immune system was otherwise found to be normal, thus excluding the possibility of defective monocyte/macrophage function that may otherwise explain our results [36]. This finding is important because mast cells have been considered to be the link between inflammation and atherosclerosis [37]. In fact, cardiac mast cells are found activated in the coronary arteries and the heart in association with atherosclerosis [23,24]. Mast cells are well known for their involvement in allergy and 'late phase' reactions by virtue of their release of different pre-stored or newly synthesized mediators [38]. It is now evident that mast cells also release many

cytokines, including IL-6 [22] that is synthesized de novo upon stimulation. Mast cell-derived IL-6 and TNF-α induce the expression of vascular adhesion molecules that participate in the pathophysiology of inflammatory heart disease [39]. Neurogenic stimulation of cardiac mast cells by stress [21] is further supported by the recent demonstration that adventitial mast cells were in contact with sensory nerve fibers in human atherosclerotic coronary arteries [40]. As a result, the mast cell is now considered as an important effector cell in neuroinflammatory conditions [21] with a versatile role not suspected previously [41].

Theoretically, IL-6 could also come from mast cells in other organs, such as the skin where the number of mast cells is high. In view of the fact that IL-6 is synthesized de novo, we could not show the contribution of cardiac mast cells as we had previously done with histamine [27]. We previously reported that cromolyn used as herein inhibited cardiac mast cell degranulation, but the stress duration was only 30 min [26]. Our observation that cromolyn only weakly inhibited stress-induced IL-6 release in both C57BL and ApoE k/o mice after 2 h of stress could be due to: (a) selective IL-6 release from mast cells without degranulation [42] that is not inhibited by cromolyn (unpublished data); (b) possible tachyphylaxis with cromolyn; and (c) release of IL-6 from other sources, for instance cardiomyocytes [43,44].

Here we showed that the number of cardiac mast cells was greater and more were located perivascularly in ApoE k/o mice than in C57BL mice, even though the extent of mast cell activation due to restraint stress was about the same. We had previously shown that acute restraint stress induced mast cell activation in the rat and mouse [26,27] heart, an effect inhibited by pretreatment with a CRH neutralizing antibody, or the CRH receptor antagonist Antalarmin [26]. CRH [45] and Ucn [46] were also shown to directly trigger mast cell activation in rat skin, and human leukemic mast cells were shown to express mRNA for CRH-R [45]. Therefore, Ucn could induce cardiac mast cells directly to release IL-6. The inhibition of stress-induced IL-6 release by pretreatment with the non-selective CRH-R antagonist Astressin [47] indicates that peripheral CRH/Ucn is involved. Ucn, which shares 45% amino acid homology with CRH, has high affinity for CRH-R2 [8], especially CRH-R2β which is predominantly expressed in peripheral tissues including the heart [48], and the expression of Ucn in the heart has been documented [49]. The higher release of IL-6 in CRH k/o mice suggests that: (a) the lack of CRH may be overcompensated by the availability of more Ucn, which is also more potent than CRH in stimulating IL-6 release; this premise is supported by the study conducted by Ando et al., who showed that intraperitoneal Ucn caused more IL-6 release than CRH in rats [14]; (b) corticosterone produced by HPA axis activation may inhibit IL-6 release, in which case CRH k/o animals escape this inhibition; however, in adrenalectomized mice supplemented with corticosterone,

the response of IL-6 release to stress was reduced rather than augmented [50], indicating that at least corticosteroids could not affect stress-induced IL-6 release. CRH deficiency was reported to impair, but not block, the response of the HPA axis to various stressors [51], implying that there was some other compensatory mechanism. In fact, IL-6 could be an essential, CRH-independent activator of the HPA axis [52].

Even though mast cells appeared to be necessary for the release of IL-6 to acute stress, serum IL-6 could also be coming from other sources, such as cardiomyocytes. It was recently shown that cardiomyocytes are capable of releasing IL-6 under certain circumstances, including hypoxia [53]. In addition, we have found that cardiomyocytes respond to Ucn with increased production of IL-6 through the activation of CRH-R2 and subsequent MAP kinases and NF-κB [54].

Increasing evidence implicates focal inflammation in the pathophysiology of CAD [12,33,34]. Acute stress is well known to worsen [3,4,55] or precipitate cardiac ischemia, especially in patients with CAD [2–6,56]; IL-6 is elevated in patients with ACS and is expressed in atherosclerotic lesions of both mice and humans. In fact, it is considered a possible link between inflammation, stress and CAD [19]. The current findings may help us better understand stress-related inflammatory conditions, particularly CAD and ACS, in which intracoronary IL-6 release was documented recently [12].

Acknowledgements

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