Acute Stress Results in Skin Corticotropin-Releasing Hormone Secretion, Mast Cell Activation and Vascular Permeability, an Effect Mimicked by Intradermal Corticotropin-Releasing Hormone and Inhibited by Histamine-1 Receptor Antagonists

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Key Words
Azelastine · Calcium · Corticotropin-releasing hormone · Mast cells · Olopatadine · Skin · Urocortin

Abstract
Background: Mast cells play an important role in allergic inflammation by releasing vasoactive molecules, proteases and cytokines. Corticotropin-releasing hormone (CRH) and its structural analogue urocortin (Ucn) were shown to trigger skin mast cell activation and vascular permeability. We investigated the effect of acute stress on rat skin vascular permeability and CRH secretion, as well as the effect of intradermal CRH, and that of two histamine-1 receptor antagonists, azelastine and olopatadine, on vascular permeability. Methods: Rats were stressed by restraint and vascular permeability was assessed by extravasation of $^{99}$Tc-glueceptate, while mast cell activation was determined by skin rat mast cell pro-
tease-1 (RMCP-1) content. Skin CRH content was evaluated by ELISA. The effect of intradermal injection of CRH and Ucn, as well as that of two histamine-1 receptor antagonists, azelastine and olopatadine, was assessed by Evan’s blue extravasation. Purified rat peritoneal mast cells (RPMCs) were also pretreated with azelastine (24 μM) or olopatadine (133 μM) for 5 min before challenge with compound 48/80 (0.5 μg/ml) for 30 min. Histamine secretion was measured fluorometrically. Intracellular Ca$^{2+}$ ions were evaluated in RPMCs loaded with calcium crimson and stimulated with compound 48/80. Results: Acute stress increased skin vascular permeability and CRH content, while it decreased RMCP-1. Intradermal injection of CRH or Ucn induced substantial Evan’s blue extravasation that was inhibited by pretreatment with azelastine (24 μM) and olopatadine (133 μM). Both antihistamines also inhibited histamine release and intracellular increase of Ca$^{2+}$ ions from RPMCs stimulated by compound 48/80. Conclusions: These results indicate that acute stress increases skin CRH that can trigger mast cell-dependent vascular permeability, effects inhibited by certain histamine-1 receptor antagonists, possibly acting to reduce intracellular Ca$^{2+}$ ion levels.
Introduction

Mast cells play an important role in allergic reactions through IgE and antigen-dependent release of histamine, leukotrienes, prostaglandin D2 and proteases [1, 2]. Mast cells also secrete numerous proinflammatory cytokines that may contribute to chronic allergic diseases [2–5]. Acute stress was recently shown to activate skin mast cells [6], but its effect on vascular permeability had not been investigated. Intradermal corticotropin-releasing hormone (CRH) [7] and urocortin (Ucn) [8] were previously shown to activate rat skin mast cells and lead to mast cell-dependent vascular permeability. Increased vascular permeability due to intradermal injection of CRH was also recently confirmed in humans [9]. However, there have been no reports on whether stress could increase the skin content of CRH. As increased vascular permeability may be involved in stress-induced skin diseases [6], it appeared reasonable to test whether clinically available drugs could inhibit CRH-induced vascular permeability.

Certain histamine-1 receptor antagonists have been reported to inhibit the release of mast cell mediators [10, 11]. Azelastine is such an antihistamine present in the ophthalmic solution Optivar® and has been shown to inhibit the release of inflammatory mediators in asthma and nasal allergy [12, 13], as well as of histamine from rat peritoneal mast cells [14]. Olopatadine is another antihistamine found in the ophthalmic solution Patanol® and has been reported to inhibit human conjunctival mast cells [15, 16]. We investigated the effect of these antihistamines on CRH-induced skin vascular permeability and nonallergenic activation of rat peritoneal mast cells (RPMCs), for the possibility they may be helpful in stress-induced skin disorders [17]. Here, we report that acute stress led to elevated skin CRH content in areas of increased vascular permeability; this effect was mimicked by intradermal CRH administration and was inhibited by azelastine and olopatadine.

Materials and Methods

Azelastine hydrochloride ophthalmic 0.05% solution (Optivar®) was provided by Asta Medica/Muro Pharmaceuticals Inc. (Tewksbury, Mass., USA), and olopatadine hydrochloride ophthalmic 0.1% solution (Patanol®) was from Alcon Laboratories, Inc. (Fort Worth, Tex., USA). The molarity of the stock concentrations of these histamine-1 receptor antagonists in the respective ophthalmic solutions were 1,195 and 2,670 μM. The concentrations used for the rat skin experiments were 24 and 60 μM azelastine, or 133 μM olopatadine. We had determined that 24 μM azelastine and 133 μM olopatadine gave equivalent inhibition of mast cell activation [18]. Compound 48/80 (Sigma, St. Louis, Mo., USA) at a final concentration of 0.5 μg/ml and disodium cromoglycate (cromolyn) at a final concentration of 100 μM (Sigma) were dissolved in 0.9% NaCl (normal saline).

RPMC Purification

Rats were sacrificed with carbon dioxide asphyxiation. The peritoneal cavities were lavaged with complete Locke’s buffer. Cells were purified over 22.5% metrizamide in Locke’s buffer and were collected by centrifugation and the histamine was then measured in the supernatant fluid and pellet using a luminescence spectrometer (Perkin-Elmer, Norwalk, Conn., USA) [20].

Evan’s Blue Extravasation

Male Sprague-Dawley rats, each weighing approximately 350 g (Charles River, N.Y., USA) were housed 3 per cage, provided with food and water ad libitum and maintained on a 14:10 h light:dark cycle. Each animal was handled one at a time in order to minimize stress. Rats were anesthetized with a single intraperitoneal (i.p.) injection of 0.25 ml ketamine and xylazine (100 mg/ml each). They were injected intravenously (0.6 ml for rats) via the tail vein with 1% Evan’s blue 10 min after intradermal injection of the test substances; this approach reduced the background extravasation. Experimental molecules were then tested by intradermal injection in normal saline 0.9% NaCl using a tuberculin syringe. The pretreatment solutions of azelastine (24 μM) or olopatadine (133 μM), when appropriate, were injected first and allowed to remain in the skin for 5 min; compound 48/80 (0.5 μg/ml), CRH or Ucn (10 μM) was then injected in the same spot with a different syringe; normal saline was injected as control. The animal was killed 15 min later by asphyxiation over CO2 and decapitated; the abdominal skin was removed, turned over and photographed. Identical circular skin areas (1 cm²) were then cut out of the samples and pellet using a luminescence spectrometer (Perkin-Elmer). The dye was measured fluorometrically (excitation wavelength of 620 nm and emission of 680 nm) as described previously [9].

Restraint Stress and Extravasation of 99Tc-Gluceptate

Rats were stressed for 30 min by placing them into an immobilizer and skin pieces were obtained as described above. Adjacent sections were sampled for 99Tc-glucetate extravasation, as well as skin rat mast cell protease-1 (RMCP-1) and CRH content. The animals were brought into a quiet procedure room within the animal facility one at a time and were allowed to walk around on the bench, where the Plexiglas immobilizer (Harvard Apparatus, Cambridge, Mass., USA) was located, for a few minutes at the same time (9–11 a.m.) every day for 5 days to ‘familiarize’ them with handling outside the cages. On the day of the experiment, each rat (if not a control) was kept in the immobilizer for 30 min. The control animal was left in its cage on the bench for an equivalent amount of time, but always at least 60 min after all procedures with the experimental animal are over. At the end of the stress period (or the wait period for the con-
Control 4 67,728 ± 14,669 0.007 94.7 ± 28.3 19.9 ± 8.1
Restraint stress (30 min) 4 176,413 ± 29,613* 0.103* 16.7 ± 2.4* 328.6 ± 45.8*
Compound 48/80 3 123,814 ± 17,980* – 42.4 ± 3.0* N/A
Stress vs. control* 4 p = 0.002 p = 0.001 p = 0.001 p = 0.001
Compound 48/80 vs. control* 4 p = 0.002 – p = 0.024 N/A

/* Using the Mann-Whitney U test.

Table 1. Effect of acute stress on rat skin vascular permeability, CRH level and mast cell activation

Experimental conditions n Vascular permeability 99Tc, cpm/100 mg Skin CRH ng/ml Mast cell activation residual RMCP-1 ng/ml Serum corticosterone ng/ml

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Intracellular Ca2+ Ion Measurement
RPMCs were incubated with 50 μM calcium crimson probe (Molecular Probes, Eugene, Oreg., USA) for 8 min at 37 °C, washed free of dye and then treated with compound 48/80 (0.5 μg/ml). The fluorescence intensity was evaluated at 10, 20, 30, 40, 690, 700, 710 and 720 s during mast cell activation using confocal microscopy and notably increased over time [22]. RPMCs were also pretreated with azelastine (24 μM) or olopatadine (133 μM) for 5 min prior to stimulation with compound 48/80 (0.5 μg/ml) and the fluorescence intensity was again evaluated for the same duration.

Statistics
Results are presented as the mean ± standard deviation. Significance was evaluated by ANOVA for histamine release and by the non-parametric Mann-Whitney U test for skin 99Tc, RMCP-1 and CRH content, and was set at p < 0.05.

Results
Effect of Acute Restraint Stress on Rat Skin Mast Cell Activation and Vascular Permeability
In this study, 99Tc levels were significantly increased in tissues of rats (n = 4) restrained for 30 min (176,413 ± 29,613 cpm/100 mg tissue) when compared with control (n = 4) rats (67,728 ± 14,669 cpm/100 mg tissue) as shown in table 1 (p = 0.002). Compound 48/80 (0.5 μg/ml i.v.) used as positive control by stimulating mast cells (n = 3) increased 99Tc extravasation to 123,814 ± 17,980 cpm/100 ng (table 1).
**Effect of Acute Stress on Skin CRH Content**

Serum corticosterone level significantly increased (p = 0.001) from 19.9 ± 8.1 ng/ml (control group) to 328.6 ± 45.8 ng/ml in restraint-stressed rats (table 1), indicating the rats were stressed adequately. Acute stress for 30 min significantly increased (p = 0.001) CRH content from 0.007 to 0.103 ng/ml after stress in skin areas adjacent to those sampled for 99Tc extravasation (table 1).

**Effect of Acute Stress on Skin Mast Cell Activation**

Skin RMCP-1 level decreased (p = 0.024) in rats following restraint stress for 30 min from 94.7 ± 28.3 to 16.7 ± 2.4 ng/ml in control rats (table 1). This result indicates that skin mast cells were activated and secreted RMCP-1.

**Effect of Azelastine and Olopatadine on Rat Skin Evan’s Blue Extravasation**

Injection of compound 48/80 (0.5 μg/ml), used as positive control, as well as of CRH or Ucn (10 μM), induced substantial Evan’s blue extravasation (left) as compared to normal saline (right) (fig. 1a) indicating mast cell activation. The extent of dye extravasation due to CRH or Ucn was equivalent to that of compound 48/80. However, when the site was pretreated with azelastine (24 or 60 μM) or olopatadine (133 μM) for 5 min before injection of compound 48/80 (fig. 1a), CRH (fig. 1b) or Ucn (fig. 1c) the extravasation was completely inhibited (left). Injection of the drugs alone (right) had no effect. Notably, cromolyn (100 μM) did not prevent extravasation (fig. 1a). These subjective results were confirmed with objective assessment of Evan’s blue extracted in formamide and measured fluorometrically. The inhibition exerted by 24 μM azelastine was equivalent to that seen with 133 μM olopatadine (fig. 2).

We then studied the effect of compound 48/80, CRH and Ucn on activation of PRMCs; we also investigated the effect of azelastine and olopatadine on this process. This approach was undertaken because acute stress, compound 48/80, CRH and Ucn were shown previously [6, 7] and here to activate skin mast cells.

**Effect of Azelastine and Olopatadine on Histamine Release from PRMCs**

PRMCs stimulated with compound 48/80 (0.5 μg/ml) for 30 min at 37°C, secreted 27.6 ± 1.6% histamine (p < 0.05, fig. 3). Neither CRH nor Ucn (10 mM) could stimulate histamine release (results not shown). Azelastine (24 μM) or olopatadine (133 μM) significantly (p < 0.05)
Fig. 2. Effect of azelastine and olopatadine on compound 48/80 (a), CRH (b) and Ucn (c)-induced rat skin Evan’s blue extravasation (n = 8). The skin was pretreated intradermally with azelastine (24 µM) or olopatadine (133 µM) for 5 min before injection of Evan’s blue (1%) in the tail vein, followed by compound 48/80 (0.5 µg/ml), CRH (10 µM) or Ucn (10 µM) intradermally for 10 min. Skin was removed and the extravasated Evan’s blue-associated fluorescence was measured after extraction in formamide. Compound 48/80, CRH or Ucn alone induced significant increases in fluorescence (p < 0.05).

Effect of Azelastine on Intracellular Ca²⁺ Ion Level in RPMCs

RPMCs loaded with calcium crimson probe for 8 min, washed and then treated with compound 48/80 (0.5 µg/ml) increased their intracellular Ca²⁺ level as was demonstrated by increased fluorescence intensity (fig. 4a). Neither CRH or Ucn (10 µM) induced any fluorescence increase (results not shown). In contrast, RPMCs pretreated with azelastine (24 µM) for 5 min and then stimulated with compound 48/80 showed almost no increase in fluorescence intensity (fig. 4b). Similar results were seen with 5 times higher concentrations of olopatadine (results not shown). These results indicate that azelastine inhibited compound 48/80-induced histamine release to about the same extent (fig. 3).

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Discussion

Here we report for the first time that acute restraint stress increased skin vascular permeability, as well as skin CRH levels, an index of local stress. The increase in skin CRH under stress indicates that, in addition to release of hypothalamic CRH, local release of CRH occurs and may have proinflammatory effects, as suggested previously [6]. Increased serum corticosterone levels, an index of HPA axis activation, indicated that the rats were stressed as the HPA axis was activated sufficiently, as shown previously [21]. Had the extravasation results been negative in the absence of any serum corticosterone elevation, one may have concluded that the skin response was negative because the rats had not been stressed enough. The RMCP-1 increase in skin areas adjacent to those showing CRH elevation indicates that skin mast cells had degranulated and released their RMCP-1, leading to decreased content. This finding is supported by our present and previously reported [6, 8] findings that intradermal administration of CRH, or its structural analogue Ucn, induced skin vascular permeability. A similar effect was recently reported in humans [9].

Neither CRH nor Ucn could stimulate histamine release or increase intracellular Ca\(^{2+}\) ion levels in RPMCs. A possible explanation could be that skin mast cells express CRH receptors, while peritoneal mast cells do not. This possibility is supported by recent reports that skin [23] and articular [24] mast cells express CRHR-1. Mast cells may express CRH only in tissues that are innervated especially by CRH- or Ucn-containing nerves, an unlikely possibility in the peritoneal cavity. Another explanation is that CRHR are stripped from RPMCs during the purification process, especially if they are few in numbers.
Here, we also report that pretreatment with azelastine or olopatadine inhibited vascular permeability induced by intradermal CRH or Ucn. Similar inhibition was noted for histamine release from RPMCs challenged with compound 48/80. Azelastine was about five times more potent than olopatadine in blocking rat skin vascular permeability and histamine release. Furthermore, azelastine and olopatadine inhibited intracellular Ca²⁺ ion levels, typically induced by compound 48/80. Such inhibition had been previously reported for the inhibitory effect of chondroitin sulfate [22]. Azelastine has been reported to inhibit the allergic response [25, 26], as well as the release of inflammatory mediators [27–30] and histamine from rat mast cells following immunologic and nonimmunologic stimuli [14]. Azelastine also inhibited tryptase and IL-6 release from immunologically challenged normal human mast cells [18], while olopatadine prevented mediator release from human conjunctival mast cells [15, 16, 30].

Cromolyn had little effect on skin vascular permeability in our studies. Even though cromolyn is known to inhibit RPMC secretion [31], it does not inhibit human lung mast cells [32] or cultured human mast cells, except at much higher concentrations [25].

Certain histamine-1 receptor antagonists may, therefore be of help in stress-induced skin disorders.

Our results support previous studies indicating that acute stress worsens skin disorders [32] and upregulates CRH-2β receptor expression in areas of stress-induced alopecia [33]. The skin has been proposed to have a local HPA axis [34] and skin mast cells have been hypothesized to act as ‘universal sensors of environmental and emotional stress’ [35]. In fact mast cells are now considered as a major regulator of neuroimmunoendocrine disorders [36].

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References