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Biochemical Pharmacology 66 (2003) 331–342
Biochemical Pharmacology
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Histamine releasing peptide (HRP) has proinflammatory effects and is present at sites of inflammation

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Received 15 October 2002; accepted 21 March 2003

Abstract

Albumin, the most abundant plasma protein, readily enters sites of inflammation during the period of increased vascular permeability. There it encounters proteases released from mast cells and invading leukocytes which earlier work has shown can act on albumin to liberate the peptide, histamine releasing peptide (HRP), first identified and named by its ability to stimulate histamine release from isolated mast cells. In this report we show that HRP releases histamine from cutaneous mast cells *in vivo* resulting in increased vascular permeability and persistent edema while *in vitro*, HRP promotes chemotaxis of leukocytes and enhances macrophage phagocytosis. Moreover, we show that the level of HRP is increased with the induction of an acute cutaneous inflammatory response in rats, that HRP is present at sites of acute and chronic inflammation in humans and that HRP is rapidly degraded by proteases thereby limiting its action to the area of its generation. We suggest that HRP is a pro-inflammatory peptide that helps amplify and perpetuate the inflammatory response. Inhibitors of inflammatory proteases or antagonists that block the action of peptides like HRP may, therefore, be useful in breaking the cycle of inflammation.

Keywords: Inflammation; Protease generated peptide; Albumin-derived peptide; Chymase; Mast cell

1. Introduction

The liberation of peptide signals that are contained within larger proteins by secreted proteases is well established [1,2]. Examples include bradykinin cleaved from kininogen by kallikrein [3], angiotensin generated from angiotensinogen by the combined action of renin and converting enzyme [4], angiostatin cleaved from plasminogen by metalloelastase (MMP-12) [5] and endostatin cleaved from collagen by cathepsin L and matrix-metalloproteases [6]. These systems appear to operate by the rapid activation or release of the protease, initiating the formation of signaling peptide(s) from an extracellular pool of substrate. These particular signaling peptides have roles in regulating the vascular tone and vascular responses to inflammation.

The inflammatory response is characterized by an initial period of hyperemia accompanied by an increase in vascular permeability and an extravasation of plasma. This is followed by the emigration of leukocytes to the inflammed area which, along with tissue mast cells, secrete a variety of inflammatory mediators including the acid proteases, cathepsin D and E [7,8]. The abundance of albumin in plasma led us to ask whether albumin contains biologically active peptide sequences that could be liberated by acid proteases. Using pepsin to mimic cathepsins D and E, we isolated an eight amino acid peptide (I-A-R-R-H-P-Y-F) from digests of mammalian albumins and, based on this peptide's ability to stimulate histamine secretion from isolated mast cells, we named it HRP [9]. Showing that HRP could be cleaved from albumin by proteases secreted by stimulated rat mast cells [10], rat peritoneal macrophages [11] and rat neutrophils [12], we hypothesized that HRP might be formed during the inflammatory response and that it might serve to amplify and perpetuate inflammation [10].

In the work reported here, we show that HRP can stimulate mast cells *in vivo*, can activate isolated neutrophils and

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Abbreviations: HRP, histamine releasing peptide; REA, radioenzymatic assay; HRF, histamine releasing factors; NT, neurotensin; SP, substance P.

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doi:10.1016/S0006-2952(03)00251-X

macrophages and is present at sites of inflammation in both rats and humans. HRP is rapidly degraded by plasma proteases, which could provide a means of localizing its effects and preventing systemic reactions. A preliminary account of some of this work has been reported [13]

2. Materials and methods

2.1. Materials

All chemicals were from Sigma-Aldrich, unless otherwise noted and radioisotopes were from New England Nuclear. Rats were from Charles River Laboratories and mice were from The Jackson Laboratory. Animals were housed in accordance with NIH guidelines and all protocols involving animals, were approved by the Tufts University Medford campus Institutional Animal Care and Use Committee.

2.2. Cutaneous vascular permeability

Rats (male, Sprague–Dawley, 250–350 g), were anesthetized (sodium pentobarbital, 25 mg/kg), the abdomen shaved, Evans Blue dye (0.1 mL/100 g body wt of a 1% solution in isotonic saline) injected as previously described [14] and 10 min later, HRP or saline (0.1 mL) injected intradermally. At some sites, diphenhydramine HCl (20 μg/mL), a histamine-receptor type I antagonist, was given immediately before the HRP. After 10 min, the animal was killed by decapitation, the abdominal skin removed, and the sites of injection visually assessed for intensity of Evan Blue staining by two observers blind to the experimental protocol. The intensity of staining was assigned a number from 0 to 5 (0 = uninjected site; 5 = maximum intensity of staining). The intensity of staining is proportional to the increase in vascular permeability [14]. The means ± SEMs were then calculated from several experiments and the results plotted as increase in vascular permeability (relative units) vs. the dose of HRP injected.

2.3. Skin blisters

''Blisters'' were raised on the shaved skin surface of an anesthetized rat *via* the intradermal injection of a small volume (100 μL) of Locke solution, without BSA as previously described [15]. Locke solution (100 μL) containing various concentrations of HRP or HRP plus disodium cromoglycate (DSCG, 100 μM), an inhibitor of mast-cell secretion [16], were then injected into the ''blister.s'' Locke solution alone (100 μL) was injected as the negative control. Ten minutes later, a small (~30 μL) sample was withdrawn from the ''blister,'' centrifuged at 4° at 10,000 *g* for 3 min, and the supernatant fraction immediately frozen for determination of histamine by radioenzymatic assay (REA) [15,17].

2.4. Mouse-ear edema

Mice (mast-cell deficient ($WBB6F_1$ W/W^v and their normal counterparts, $WBB6F_1{}^{+/+}$) were anesthetized and the thickness of the pinna of each ear measured (three measurements per ear) using a micrometer [18,19]. HRP (20 μL of a 200 μM solution) was then injected into the left ear, the right ear serving as a buffer injected control. Swelling was then measured by micrometer (the mean ± SEM of three measurements/time point/ear/mouse) at 2, 4, and 6 hr after the injection. The difference between the mean baseline value (i.e. before injection) for each ear and the mean value measured at various times after the injection of HRP or saline was then determined. The results from four mice in each group were then averaged. The mean difference (change in edema) was then plotted vs. time for the saline and HRP injected ears for the normal and mast-cell deficient mice.

2.5. Chemotaxis of human and rat leukocytes

Chemotaxis was measured using a standard blind-well, Boyden chemotactic chamber (Nucleopore) and the leading-edge assay procedure [20–23]. The upper and lower chambers were separated by polycarbonate filters of pore size 5 μm for neutrophils and 8 μm for macrophages [23]. Rat peritoneal neutrophils or macrophages were obtained 4 or 48–72 hr, respectively, after the induction of sterile peritonitis as previously described [11,12,24]. Human peripheral neutrophils were isolated from peripheral blood drawn by venapuncture by trained phlebotomists from healthy volunteers after informed consent. The protocol for drawing blood was approved by the Human Studies Review Board at Tufts University. Erythrocytes were removed by hypo-osmotic lysis and the neutrophils separated by centrifugation on Percoll gradients [25]. The cells (2×10^5–4×10^5 mL^{-1}) were then suspended in Hank's buffer containing 10 μg/mL polymyxin B to eliminate endotoxin contamination, and placed in the upper chamber. Various concentrations of HRP were added to the lower chamber, thereby introducing a gradient of peptide from the upper to the lower chamber. After incubation at 37° for 30 min, the filters were removed, fixed, stained (Diff Quick, Fisher) and the distance to the leading edge of the migrating cells measured in 10 fields using an ocular micrometer under bright field microscopy at 200× magnification. Each concentration of HRP was used in triplicate and the experiment repeated three times. The mean ± SEM migration distances were then calculated.

2.6. Macrophage phagocytosis

Peritoneal macrophages were isolated from rats 48–72 hr after the induction of sterile peritonitis, resuspended in RPMI 1640 culture media (10^7 cells/mL), and incubated in 24-well Falcon culture plates for 2 hr at 37°. Non-adherent

cells were then removed and fresh RPMI media added. Phagocytosis was assayed by measuring the uptake of ^{51}Cr-labeled opsinized (with fresh rat serum, 1:10 dilution) sheep erythrocytes (ShEA, Cappel Laboratories) [26] at a 1:100 macrophage to ShEA ratio. HRP (in varying concentrations) or media alone (control) was then added followed by the addition of ^{51}Cr-labeled ShEA and the incubation continued at 37° for 1 hr. The supernatant was then carefully removed, non-phagocytosed labeled-ShEA lysed using NH_4Cl, the macrophages washed three times, dissolved with 0.5% SDS and aliquots counted for ^{51}Cr-radioactivity. Each concentration of HRP and controls were run in triplicate and the experiment was repeated three times and the results averaged. The results (phagocytosis) are expressed as the percent increase above control (media alone) values.

2.7. Synovial and friction blister fluid samples

Blood and synovial fluids from patients with rheumatoid arthritis (four females, three males, mean age = 66 years) were collected after informed consent into 4° heparinized tubes or tubes containing an inhibitor cocktail, respectively. The tubes were then centrifuged at 4° to separate plasma and remove cells from synovial fluids and the plasma and cell-free synovial fluid samples immediately frozen as previously described [27]. The samples were then extracted and subjected to reverse-phase HPLC on micro-Bondapak C18 columns prior to measurement of HRP by RIA [10]. HRP activity eluting at the position of the synthetic standard was integrated and expressed as fmol HRP/mL original fluid. Fluid from friction blisters was obtained from healthy athletes (three males, mean age 20 years) after informed consent and was immediately frozen for later extraction and assay of HRP. These samples were not subjected to HPLC. These protocols were approved by the Human Studies Review Boards at UMass Medical School and at Tufts University.

2.8. Cutaneous inflammation and HRP measurements

Rats (male, Sprague–Dawley, 250–350 g) were anesthetized with sodium pentobarbital (25 mg/kg, because of the duration of the procedure), the abdominal skin shaved, and injected intradermally (100 μL) with croton oil (25 mg/mL in 0.1% SDS), carrageenan (25 mg/mL in saline) or saline at multiple sites. Control animals were either injected intradermally with saline or not injected. Injected sites were marked using a magic marker and 42 hr later, the animals were anesthetized (Isoflurane), immediately killed by decapitation, blood samples collected into heparinized tubes, and the skin removed. The injected sites were punched-out, weighed and rapidly homogenized on ice in 10 vol. of 0.1 N HCl containing 15 μg/mL pepstatin and extracted with 4 vol. of acid:acetone (1:3). The extracts were then separated by HPLC and the RIA activity eluting in the region of the HRP standard integrated and expressed as fmol HRP/g skin. Plasma was separated by centrifugation and was similarly extracted and assayed by RIA after HPLC.

For measurement of the degradation of HRP by plasma, HRP was added to fresh rat plasma at 37°, to plasma containing *o*-phenanthroline (1 mM) or to plasma containing a protease inhibitor cocktail (soybean trypsin inhibitor, 1 μg/mL; chymostatin, 100 μg/mL; pepstatin, 15 μg/mL; phenylmethylsulfonyl fluoride, 1 mM; and *o*-phenanthroline, 1 mM) and incubated at 37°. Samples, removed at various times, were extracted in 4 vol. of ice-cold acid:-acetone (3:100; v/v, 1 M HCl:acetone) and the reconstituted extracts were assayed for HRP by RIA [10].

2.9. Isolation and use of mast cells, mast-cell lysates and histamine assay

Mast cells were isolated from the peritoneal and pleural cavities of male Sprague–Dawley rats (300–400 g), purified to 95–98% homogeneity on 22.5% metrizamide gradients and suspended in Locke solution, as previously described [10,16]. For experiments testing the effect of disodium cromoglycate on histamine release in response to HRP, mixed populations of peritoneal and pleural mast cells were used. In experiments involving pretreatment of isolated mast cells with HRP or substance P (SP), peritoneal mast cells were used while pleural mast cells were used in those experiments involving pretreatment with neurotensin (NT). Histamine was measured by a fluorometric assay and histamine release initially expressed as a percent of total as previously described [9,16].

Mast-cell lysates were prepared by sonication of purified (95–98%) suspensions (2×10^6 mL^{-1}) of mast cells in 4° Locke's solution without BSA or glucose. The sonicates were centrifuged at 4° to remove any unbroken cells. Aliquots (0.1 mL) of lysate were then injected intradermally into the shaved abdominal skin of anesthetized (sodium pentobarbital) male rats and the injection sites marked. At various times afterward (0, 14, 30, and 60 min), the animals were killed by decapitation, skin around the injection site removed, weighed, extracted and assayed for HRP by our RIA after HPLC as described above. Levels of HRP were expressed as pmol HRP/g wet weight of skin.

For experiments on the effect of collagen on the generation of HRP by mast-cell protease, collagen (type VII, Sigma) was made as a stock solution by dissolving it in 0.01 N acetic acid at 2.5 mg/mL. On the day of the experiment, the stock solution was diluted into albumin-free Locke's solution at concentrations of 1.0 and 0.5 mg/mL and 0.5 mL of the diluted collagen in Locke's solution added to each well of 12-well culture plates. The pH of the final Locke's solution was 7.2–7.4. The plates were then incubated at room temperature for 2–3 hr. Mast cells (3×10^5–4×10^5) were then added and the incubation continued at 37° for 30 min. Compound 48/80 (1.0 μg/mL) was then added followed 5 min later by BSA to give a final albumin

concentration of 10 μg/mL. Aliquots were then withdrawn at various times afterward, centrifuged to remove any cells, and the supernant fractions extracted with 3 vol. of hydrochloric acid:acetone as described above. The extracts were then assayed for HRP by RIA [10].

Histamine from skin blister fluids was assayed by a REA as previously described [15,16]. Statistical analysis was *via* Student's *t*-test assuming unequal variances.

3. Results

3.1. Effect of HRP on cutaneous vascular permeability and edema

The ability of HRP to stimulate histamine secretion from isolated mast cells [9] suggested that this albumin-derived peptide might increase vascular permeability and produce edema *in vivo*. When given intradermally to rats, HRP caused (within 10 min) a dose-dependent increase in cutaneous vascular permeability and this response was blocked by the H-1 antagonist, diphenhydramine (Fig. 1A). To determine the time-course of any edema that might accompany the vascular permeability increase and to assess the importance of mast cells in this response, we measured swelling of the pinna in the ears of normal ($W/W^{+/+}$)and mast-cell deficient (W/W^{v}) mice. The results in Fig. 1B show that HRP produced edema between 2 and 6 hr after injection into normal ($W/W^{+/+}$) mice and that it was ineffective in mast-cell deficient (W/W^{v}) mice.

3.2. Effect of HRP on histamine release from mast cells in vivo and in vitro

The above results were consistent with the idea that mast-cell histamine mediated these effects of HRP. To test directly the ability of HRP to release histamine from cutaneous sites *in vivo*, HRP was injected into rat skin "blisters" [15]. As shown in Fig. 2A, HRP stimulated (within 10 min) a dose-dependent release of histamine into the "blister" fluid, and this effect was blocked by the inhibitor of mast-cell secretion, disodium cromoglycate. A similar dose-dependent inhibition by disodium cromoglycate was seen for the effects of HRP on isolated mast cells (Fig. 2B).

Preincubation of mast cells with 1.0 μM HRP, a concentration of HRP that did not cause any increase in release above spontaneous levels ($4 \pm 1\%$ total with HRP vs. $3 \pm 1\%$ total without HRP, mean ± SEM, N = 5) [9], caused a time-dependent decrease in histamine release elicited by the subsequent addition of 10 μM HRP (Fig. 2C). This decrease reached a plateau after 10 min of preincubation. Longer periods of preincubation did not cause any further decrease in histamine release (Fig. 2C). Preincubation with lower concentrations of HRP had no effect on the subsequent release of histamine in response to 10 μM HRP (data not shown).

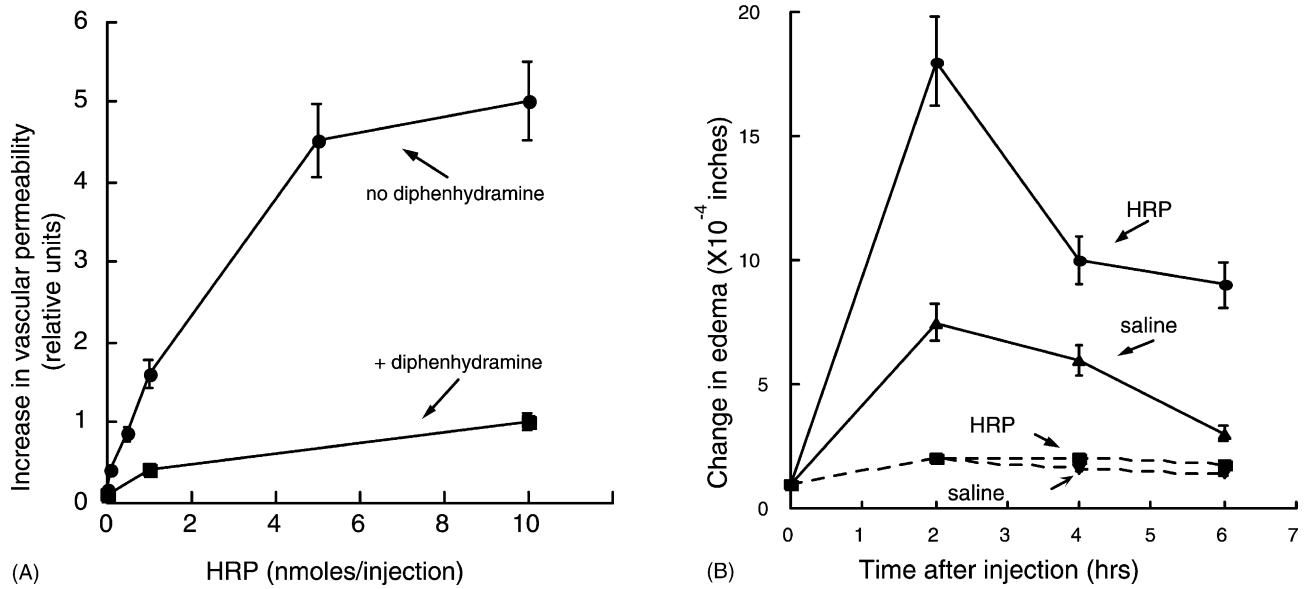

Fig. 1. Effect of HRP on (A) cutaneous vascular permeability in the rat and (B) ear edema in normal and mast-cell deficient mice. (A) HRP was injected intradermally into anesthetized rats pretreated i.v. with Evan Blue dye. After 10 min the animal was killed and the injected skin sites were visually assessed for intensity of staining using a scale of 0–5. At some sites, diphenhydramine (an H-1 receptor antagonist) was included with HRP. Diphenhydramine significantly reduced ($P < 0.005$) the increase in vascular permeability produced by HRP. Measurements of increased vascular permeability in response to >0.5 nmol HRP/site were significantly different from the control site at the $P < 0.005$ level. Mean ± SEM, N = 3. (B) HRP was injected into the left ear of anesthetized mice; the right ear was injected with vehicle alone. Swelling of each ear was measured over time and the difference between the two ears determined. HRP elicited significant edema in normal mice, but had essentially no effect in mast-cell deficient animals (– – – –). Histological analysis of injected ears showed significant neutrophil infiltration with HRP in normal mice and a reduced, but noticeable infiltration is mast-cell deficient animals. In normal mice, the values for HRP are significantly different ($P < 0.005$) from the saline injected animals. There was no significant different between the HRP and saline values for the mast-cell deficient mice. Mean ± SEM, N = 3 mice in each group.

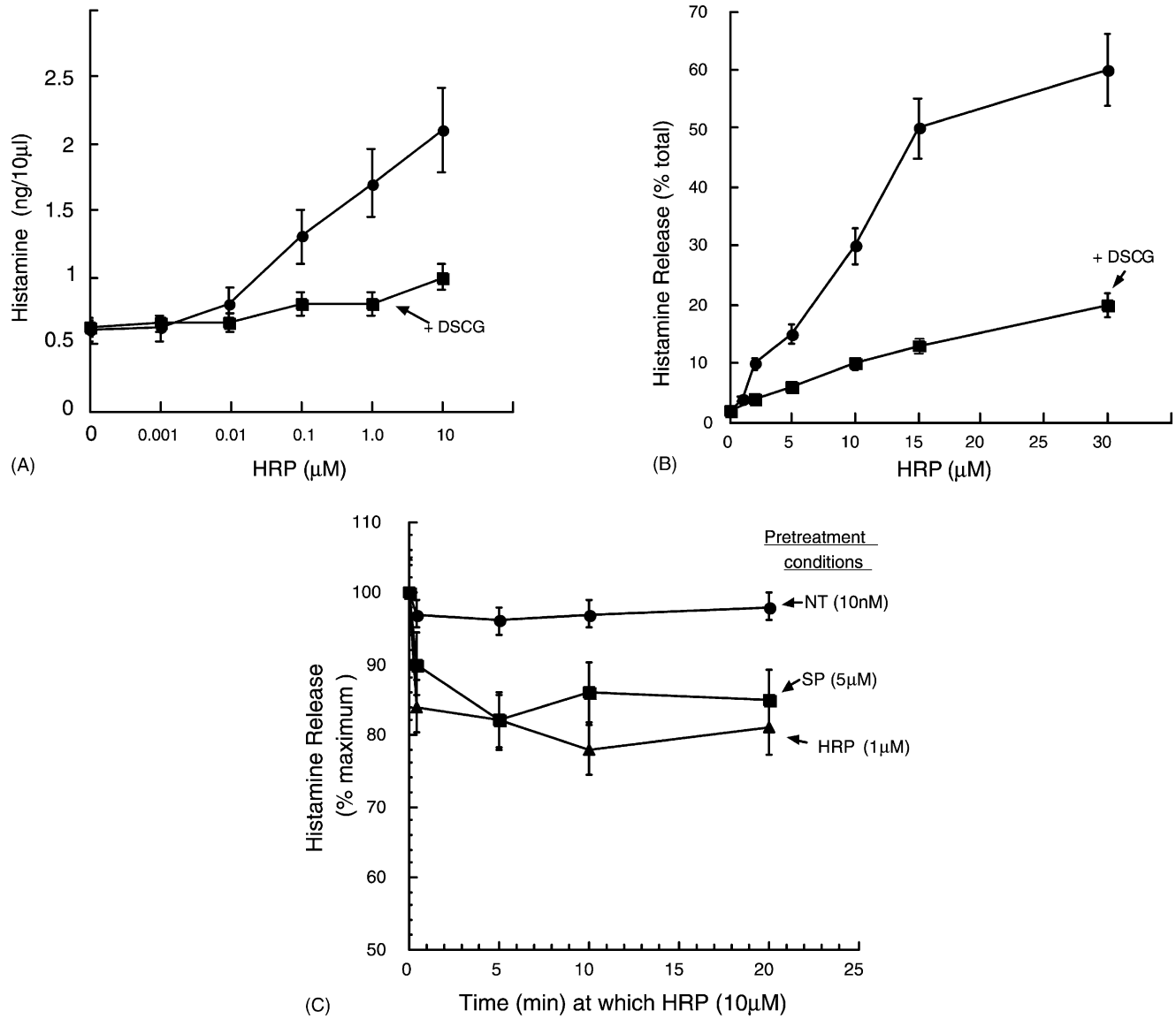

Fig. 2. Effect of HRP on histamine release from (A) skin mast cells and (B) isolated serosal mast cells in the presence or absence of the mast-cell inhibitor, disodium cromoglycate or (C) from isolated mast cells following their pretreatment with HRP, SP, or NT. (A) HRP was injected into raised skin "blisters" and aliquots withdrawn 5 min later and assayed for histamine. In some blisters, disodium cromoglycate was injected immediately before HRP. Note that HRP elicited a dose-dependent release of histamine which was prevented by disodium cromoglycate ($P < 0.01$ for HRP concentrations $\geq$0.1 uM). Mean ± SEM, N = 3 experiments. (B) HRP at the concentrations indicated, was added to suspensions of peritoneal/pleural mast cells in Locke's solution in the presence or absence of disodium cromoglycate (100 uM), incubated at 37° for 10 min, and histamine release was determined. Disodium cromoglycate inhibited histamine release ($P < 0.01$) elicited by HRP concentrations $\geq$1 uM. Mean ± SEM, N = 3. (C) HRP (10 µM) was added to suspensions of mast cells that had been pretreated for the indicated amounts of time, with either NT (10 nM), SP (5 µM), or HRP (1 µM) and histamine release determined after 10 min. Histamine release is expressed as a percent of the maximum value which occurs at time = 0 (i.e. the simultaneous addition of HRP (10 µM) and the other secretagogue). There is a significant difference ($P < 0.05$) between the time = 0 value and the values at 0.5, 5, 10 and 20 min for cells pretreated with SP or HRP. There are no significant differences ($P > 0.05$) between the values for HRP and the values for SP. Likewise, there are no significant differences ($P > 0.05$) between time points for cells pretreated with NT and then stimulated with 10 mM HRP. Mean ± SEM, N = 4 (two experiments done in duplicate).

We next tested the ability of HRP to stimulate histamine release from mast cells already activated by another peptide secretagogue. Pretreatment of mast cells with 5 µM SP, which stimulated a small but significant ($P < 0.05$) increase in histamine secretion above spontaneous levels (9 ± 2% total with SP vs. 3 ± 1% total without SP, mean ± SEM, N = 4), also produced a time-dependent decrease in histamine release elicited by the subsequent addition of 10 µM HRP (Fig. 2C). This decrease also reached a plateau after a 10 min pretreatment period. Preincubation with 1.0 µM SP, a concentration that did not increase histamine secretion above spontaneous levels (5 ± 1% total with SP vs. 4 ± 1% total without SP, mean ± SEM, N = 5) produced a smaller decrease in subsequent histamine release stimulated by 10 µM HRP; lower concentrations of SP had no effect on the subsequent stimulation (data not shown). Pretreatment of mast cells for up to 20 min with 10 nM NT [a concentration that

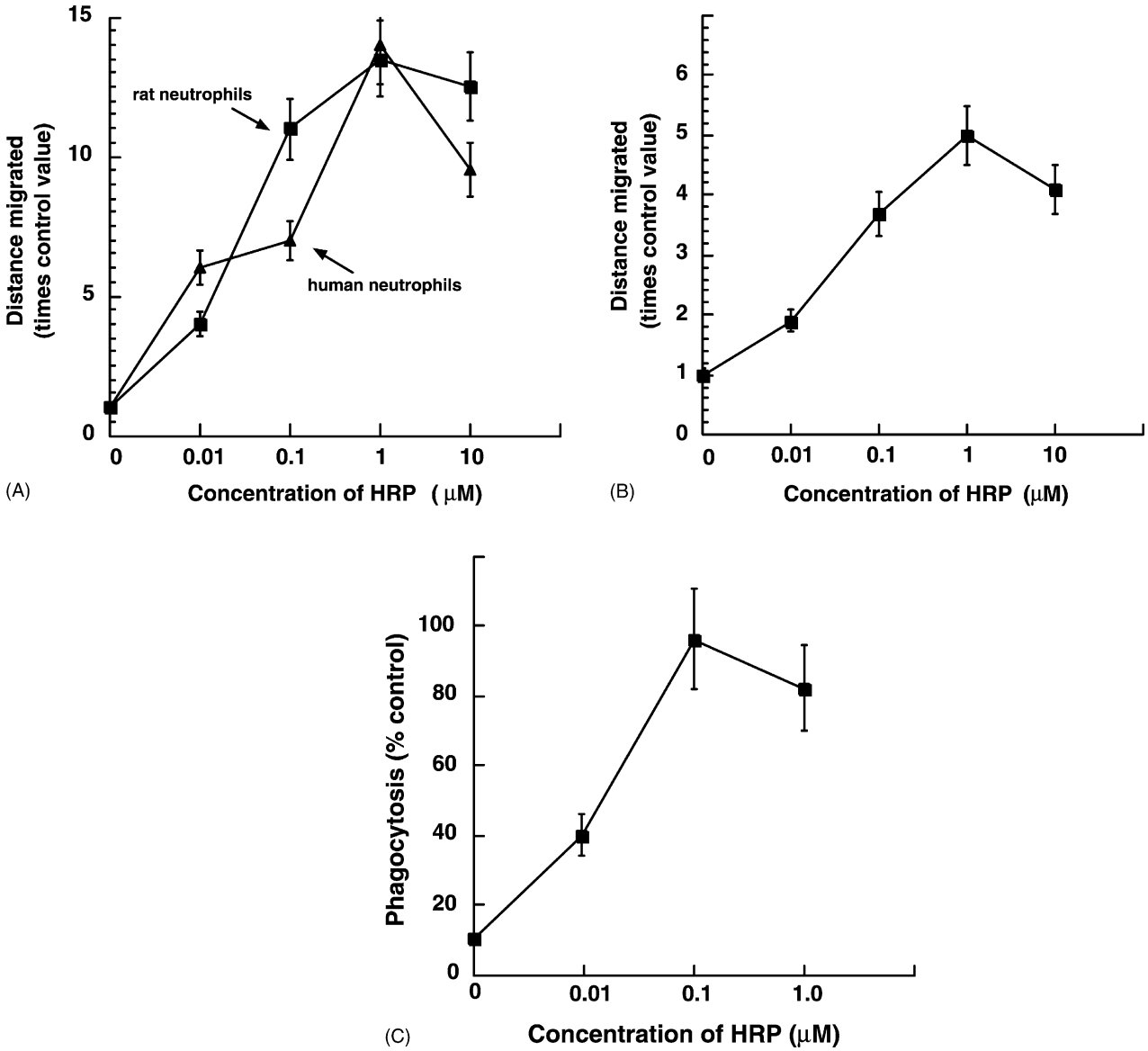

Fig. 3. Effect of HRP on (A) chemotaxis of human and rat neutrophils or (B) rat peritoneal macrophages and on (C) phagocytosis by rat macrophages. (A and B) HRP or buffer alone at 37° was added to the lower wells of Boyden chambers and suspensions of neutrophils or macrophages (1×10^5) were added to the upper chamber. After incubation at 37° for 30 min, the distance migrated by the leading edge of the cells into the filter paper was determined. Mean ± SEM, $N \geq 3$. (C) Peritoneal macrophages were incubated at 37° for 1 hr in media containing ^{51}Cr-labeled ShEA in the presence or absence of various concentration of HRP. Buffer alone was added to control cells. The incorporation of radioactivity into the macrophages was then determined. The results are expressed as the percent increase in phagocytosis above the control value. Mean ± SEM, $N = 3$.

stimulates a significant ($P < 0.05$) increase in histamine release above spontaneous levels (15 ± 2% total with NT vs. 3 ± 1% total without NT, $N = 4$)], had no effect on the subsequent release of histamine in response to 10 μM HRP (Fig. 2C). Higher concentrations of NT (50–100 nM) also had no effect on the subsequent release of histamine in response to 10 μM HRP (data not shown).

3.3. Effect of HRP on chemotaxis and phagocytosis

Using blind-well Boyden chemotactic chambers, HRP was found to stimulate, by 5- to 10-fold, the chemotaxis of isolated rat and human neutrophils (Fig. 3A) as well as rat macrophages (Fig. 3B). The EC_{50} was between 20 and 50 nM HRP. Over the same dose range, HRP enhanced by 10-fold, the phagocytosis of ^{51}Cr-ShEA by isolated rat macrophages (Fig. 3C).

3.4. Effect of inflammatory stimuli on HRP levels in skin

Stimulated mast cells, incubated with albumin, generate HRP *via* the actions of proteases such as chymase [10]. When mast-cell lysates were injected intradermally into anesthetized rats, the level of immunoactive HRP (iHRP) in skin extracts increased more than 10-fold within 1 hr (Fig. 4A).

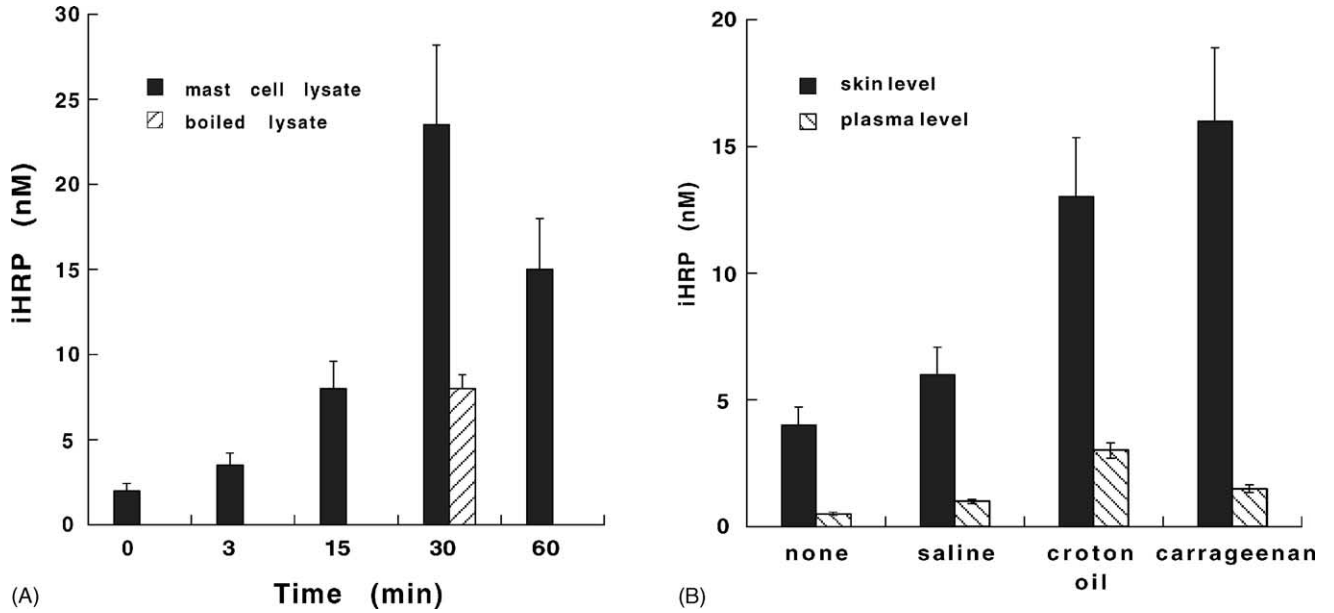

Fig. 4. HRP levels following (A) the intradermal injection of mast-cell lysates or (B) cutaneous inflammation. (A) iHRP levels in skin after the intradermal injection of mast-cell lysate. Purified mast cells (2×10^6) were lysed by sonication in 4° phosphate buffered saline and 200 μL aliquots were injected intradermally into anesthetized rats. Some aliquots of lysate were boiled for 15 min before injection. At the indicated times, skin samples were removed from the site of the injection, weighed, homogenized in acid:acetone at 4° and prepared and assayed for HRP by RIA. There was a time-dependent generation of HRP in response to the lysate and this generation was greatly reduced by prior boiling of the lysate. Mean of two experiments; bars show the range. (B) HRP levels in post-HPLC extracts of skin and plasma from rats injected intradermally 24 hr previously with saline, croton oil, or carrageenan and from uninjected controls. Skin and plasma levels of HRP from animals receiving croton oil or carrageenan were considerably higher than those from animals receiving saline or nothing. Interestingly, both the uninjected animals and the saline injected ones showed measurable amounts of HRP in skin. Mean of two experiments; bars show the range.

The increase observed using boiled mast-cell lysate was significantly less. Basal levels of iHRP were approximately 2 nM (Fig. 4A). Similarly, iHRP levels in skin were increased 4- to 6-fold, 24 hr after injection of croton oil or carrageenan (Fig. 4B). Basal iHRP levels in blood were approximately 0.3 nM and were not increased substantially during inflammation.

3.5. iHRP in arthritic synovial fluids and in friction blisters

The results in Table 1 show that iHRP levels in human synovial fluid from arthritic joints were significantly higher than in plasma obtained from the same patients. Similarly, iHRP levels were higher in fluids withdrawn from friction blisters incurred by athletes than in control plasmas.

3.6. Effect of plasma proteases on the stability of HRP

As shown in Fig. 5A, the rapid degradation of HRP by rat plasma at 37° ($T_{1/2}$, ~20 s) was slowed by the metalloprotease inhibitor, *o*-phenanthroline ($T_{1/2}$, ~40 s) and by a protease inhibitor cocktail ($T_{1/2}$, >100 s). This suggested that HRP, given in the general circulation in the absence of protease inhibitors, would not stimulate tissue mast cells. In keeping with this, intravenous injection of HRP into rats had little effect on plasma levels of histamine, while the peptide, NT (at 100-fold lower dose) elicited a rapid and significant rise in plasma histamine (Fig. 5B).

3.7. Effect of collagen on the degradation of HRP

The rapid degradation of HRP by plasma (Fig. 5A) and by mast cells [17] appeared to be at odds with our findings that nanomolar concentrations of HRP could be generated *in vivo* by inflammatory stimuli (Fig. 4B) and mast-cell lysates (Fig. 4A). This led us to hypothesize that extracellular

Table 1
Levels of iHRP in plasma, synovial fluid and blister fluid

Subject	Fluid (N)	iHRP (pmol)
Arthritic patients	Plasma (7)	$22 \pm 2^{*}$
	Synovial fluid (7)	$89 \pm 13^{*}$
Volunteer athletes	Plasma (3)	$60 \pm 6^{**}$
	Blister fluid (3)	$560 \pm 140^{**}$

Synovial fluids and plasmas were collected from patients with rheumatoid arthritis and immediately frozen. For analysis, the samples were thawed, extracted with 3 vol. of acid:acetone and the extracts subjected to HPLC. The RIA activity eluting in the region of the HRP standard was then integrated and expressed as fmol/mL original sample. Fluids from friction blisters and plasmas were collected from athletes and immediately frozen. For analysis, the samples were extracted with acid:acetone but the resulting extracts were not subjected to HPLC. The total iHRP activity in these samples is higher as it likely includes HRP and HRP-related peptides (larger fragments of N-terminally extended HRP). iHRP levels in synovial and blister fluids were significantly higher than plasma ($^{*}P < 0.01$ and $^{**}P < 0.05$, respectively). Mean ± SEM of N, number in parentheses.

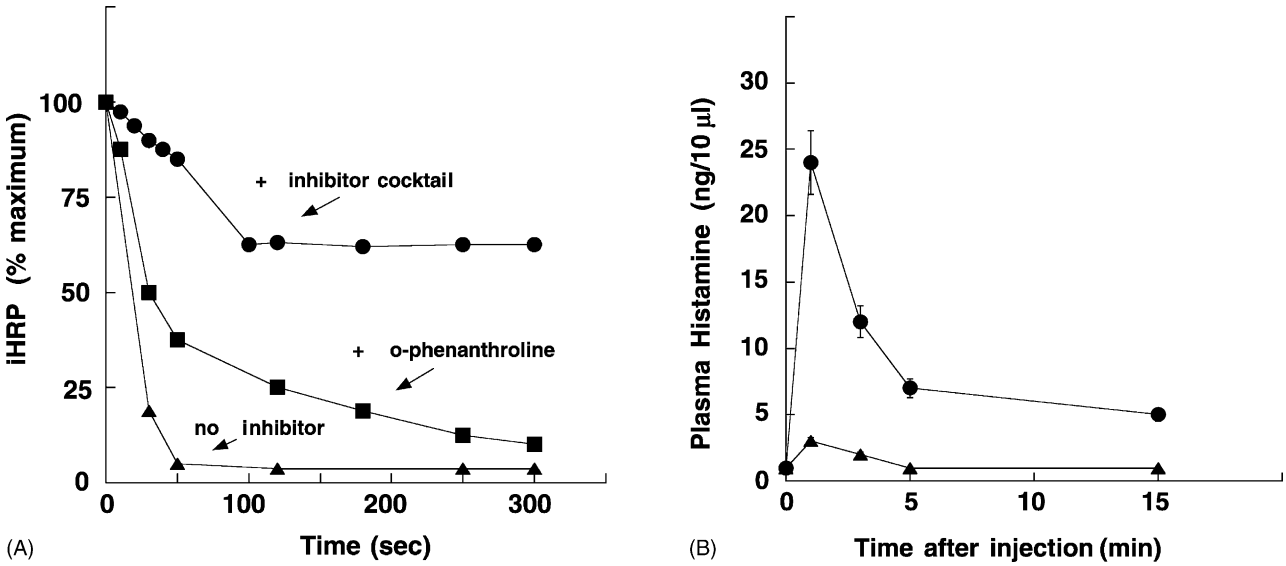

Fig. 5. Effect of protease inhibitors on the stability of HRP in plasma (A) and the effect of the intravenous injection of HRP or NT on plasma levels of histamine (B). (A) HRP (10 nmol) was added to fresh rat plasma in the presence or absence of the carboxypeptidase inhibitor, *o*-phenanthroline (*o*-phe) or an enzyme inhibitor cocktail (see Section 2). Aliquots were withdrawn over time into acid:acetone, processed and assayed for HRP. Mean of two experiments assayed in duplicate. (B) HRP (300 nmol/kg, ▲) or NT (3 nmol/kg, ●) were injected intravenously into anesthetized rats, plasma samples collected over time and assayed for histamine by REA; control animals were injected with saline. Mean of two experiments assayed in duplicate, bars show the range.

matrix proteins like collagen, that are present in the tissue environment in which inflammation occurs, might protect HRP against degradative proteases. To test this possibility, mast cells suspended in Locke's solution containing albumin (10 mg/mL) were placed in tissue culture wells containing varying amounts of collagen and stimulated with compound 48/80. iHRP was then measured over time. As shown in Fig. 6, the levels of iHRP generated were similar at 2 min after stimulation. In the absence of collagen, HRP levels decreased ($T_{1/2}$, ~10 min), whereas in presence of 0.5 mg collagen/mL, the level of iHRP remained relatively stable for 45 min.

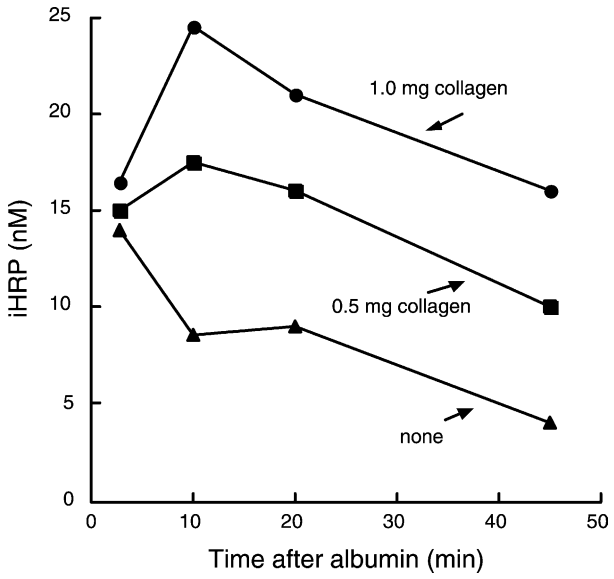

Fig. 6. Effect of collagen on the generation of iHRP from albumin by stimulated mast cells in the absence of carboxypeptidase inhibition. Collagen (type VII), at the concentrations indicated, was added to wells of a 12-well culture plate and incubated for 1 hr at 37°. Mast cells (~35,000/well) in Locke's solution without BSA were then added and after 5 min stimulated by the addition of compound 48/80 (1 µg/mL). BSA (10 µg/mL final concentration) was added 5 min later and samples withdrawn at the indicated times for measurement of HRP by RIA. Note that the presence of collagen dose-dependently increased the levels of HRP at each time point. In the absence of albumin or in the absence of mast cells, there was no generation of HRP. Mean of two experiments measured in duplicate.

4. Discussion

HRP was first identified based on its ability to stimulate the release of histamine from isolated mast cells [9]. Mast cells secrete a variety of proinflammatory molecules including cytokines such as IL-6 and TNF and significantly contribute to the development of inflammation [8,28–30]. Three lines of evidence reported here demonstrate the ability of HRP to stimulate mast cell secretion *in vivo*: (a) when injected intradermally, HRP increased cutaneous vascular permeability *via* a histamine-dependent process (Fig. 1A); (b) when injected into skin blisters, HRP elicited a dose-dependent release of histamine that was blocked by the inhibitor of mast-cell secretion, disodium cromoglycate (Fig. 2A); and (c) when injected into the pinna of mast-cell deficient mice (WBB6F, W/W^v) HRP failed to cause edema, but did so when injected into the pinna of normal (WBB6F$_1^{+/+}$) mice (Fig. 1B). These results together with our earlier findings [9], suggest that the mast cell is a target of HRP. However, while HRP stimulates the release of other preformed granule contents from mast cells in addition to histamine [9,10], whether it stimulates the synthesis and secretion of cytokines by mast cells is unknown.

The histamine releasing effect of 10 μM HRP was somewhat reduced by pretreatment of mast cells with lower concentrations of HRP or SP (Fig. 2C). This apparent ''desensitization'' of the mast-cell secretory response to HRP was slow to develop, reaching a plateau of ~20% less than the maximum level of release after a 10 min period of pretreatment. This desensitization suggests that HRP and SP may share a common binding-site on the mast cell and/or common signal transduction steps that lead to the release of histamine. While SP has been suggested to stimulate mast-cell histamine secretion in a 'receptor independent manner' by directly activating G-proteins [31], HRP's mechanism of action on the mast cell has not yet been defined.

Pretreatment of mast cells with 10 nM NT, a concentration that elicits a significant secretory response, had no effect on the subsequent release of histamine caused by 10 μM HRP (Fig. 2C). In contrast, an earlier report from our laboratory showed that pretreatment of mast cells with 10 nM NT produces a rapid and profound desensitization of the secretory response (~70% decrease in histamine release within 30 s) elicited by higher concentrations of NT (e.g. 10 μM) [32]. This earlier finding is characteristic of the desensitization seen with G-protein coupled receptors [33] and is consistent with our results showing the presence of the G-protein coupled, type1 NT-receptor on rat serosal mast cells [34,35]. Indeed, NT is the only neuroendocrine peptide for which there is pharmacological, molecular, and immunological evidence for a specific mast-cell receptor [31].

Various histamine releasing factors (HRF) have been identified in nasal washings and in incubates of human leukocytes [36]. These HRF are thought to be produced and secreted by leukocytes and range in size from 10 to ≥80 kDa. They therefore differ significantly from HRP, an eight amino acid peptide that is generated from plasma albumin by protease action.

HRP's ability to increase cutaneous vascular permeability *via* the release of histamine from skin mast cells is similar to that of other inflammatory peptides, including NT [16,17], SP [37,38], and corticotropin releasing hormone (CRH) [34]. Given intradermally at ~250 pmol/site (2.5 nM), HRP enhanced vascular permeability (Fig. 1A). Although HRP appears to be at least 50-fold less potent than these other peptides, the widespread distribution and extremely high concentration (40 mg/mL plasma, ≤1 mM) of HRP's precursor, albumin, allow for the generation of biologically active levels of HRP with minimal precursor processing (<0.01%) [least effective dose: NT = 0.15 pmol [16], SP = 2 pmol [37,38], CRH = 5 pmol [39], HRP ~250 pmol (Fig. 1A)].

Central to the inflammatory response is the emigration of leukocytes from the vascular compartment to the inflamed site along a gradient of chemoattractant molecules [40]. Once at the inflamed site, leukocytes show an enhanced phagocytotic response prompted in part by the various mediators present [41]. The ability of inflammatory proteases to generate HRP suggested that HRP might contribute to inflammation by attracting leukocytes and enhancing their phagocytic capability. Our results reported here support this idea (Fig. 3) and suggest that leukocytes are another target of HRP.

A variety of molecules have been shown to promote leukocyte chemotaxis and enhance phagocytosis [40,41]. Examples of chemotactic factors include the complement fragment C5a, leukotriene B_4, numerous chemokines, and formylated peptides like *N*-formyl-L-methionine-L-leucine-L-phenylalanine (fMLP). All these factors stimulate chemotaxis by activating G-protein coupled receptors [40,42]. Phagocytosis is enhanced by the binding of complement fragments, C3b and C4b, to CR1 and CR3 receptors while cytokines like IL-1, IL-4, and TNF-α and IgG are thought to enhance phagocytosis by actions that require Fc_ε R receptors [41]. How HRP promotes chemotaxis and enhances phagocytosis is unknown although given its size and its positive charge at physiologic pH, it seems likely that its action is exerted at the cell surface. As there is no apparent structural homology between HRP and any of these other chemoattractants, it is doubtful that HRP's action to promote chemotaxis and phagocytosis is *via* binding to any of these known receptors. HRP may therefore be acting on an as yet unidentified leukocyte receptor. Alternatively, HRP may be exerting its effect in a receptor-independent manner, reminiscent of the stimulatory action of mastoparan on mast-cell secretion [31].

Our results showing that HRP is present at sites of inflammation in rats and humans, is consistent with its proposed role as a pro-inflammatory peptide [9,10,13]. While it is possible that the HRP we measured in these samples of skin was generated during the extraction process, this seems doubtful as we used conditions which would rapidly inactivate proteases. The maximum levels of HRP we have measured (~2 nM) presumably represent steady-state levels, reflecting both the generation and degradation of HRP. Thus it seems possible that peak concentrations of HRP may reach levels sufficient to promote chemotaxis and enhance phagocytosis (i.e. ~10–50 nM, Fig. 3). The rapid degradation of HRP by plasma proteases ($T_{1/2}$, ~20 s) would most likely limit HRP's action to near the site of its generation. Indeed, HRP's short half-life in plasma most likely explains its inability to significantly elevate plasma levels of histamine when injected intravenously as compared to NT, the half-life of which in plasma is several minutes [43].

The intradermal injection of mast-cell lysate produced a time-dependent increase in the level of HRP at the site of the injection while inhibiting the enzymatic activity of chymase by boiling markedly reduced this effect. Interestingly, the intradermal injection of human mast-cell chymase into guinea pigs has been shown to produce a dose-dependent increase in vascular permeability [44,45] and inhibiting chymase's enzymatic activity also abolished

this effect. This response to chymase was slow to develop and prolonged (it was still evident 2 hr after the injection). It is tempting to speculate that this effect of chymase may be due, in part, to the generation of HRP.

The presence of low amounts of HRP in non-injected skin samples (Fig. 4B) is intriguing. This could be due to the actions of proteases spontaneously released from tissue mast cells acting on plasma albumin that has leaked into the tissue space. Alternatively, it could be due to the activation of skin mast cells that has been shown to occur when rats are subjected to acute stress [46]. Mast-cell activation would release histamine (increasing vascular permeability and promoting an influx of albumin rich plasma) and proteases (e.g. chymase). Alternatively, HRP could be contained within vesicles in inflammatory cells where it is processed from albumin. Human neutrophils and platelets have been shown to contain endocytosed albumin within their secretory vesicles [47,48] and proteins containing xenopsin, another protease generated peptide [24,49] that has both pro- and anti-inflammatory actions [24,49,50], have been recently identified in vesicle fractions from various tissues [51].

This generation of HRP *in vivo* by injected mast-cell lysate in inflammatory settings occurred in the absence of carboxypeptidase (CPA) inhibitors. This is in contrast to earlier *in vitro* work from our laboratory showing that the generation of measurable quantities of HRP by media conditioned by stimulated mast cells required the presence of CPA inhibitors [10]. This difference between the *in vitro* and *in vivo* generation of HRP may be explained by our results with collagen films (Fig. 5). They show that in the presence of collagen, no inhibition of CPA is required for stimulated mast cells to generate measurable quantities of HRP from albumin. There are several possible mechanisms by which collagen could enable HRP-generation to occur. Collagen could "protect" HRP from degradation by acting as a competing substrate or collagen-derived peptides could inhibit the degradative proteases. It is also possible that collagen enhances the generation of HRP by stimulated mast cells. We have shown that collagen does not contain an HRP-like peptide and that collagen alone cannot generate HRP from albumin. Finally, our earlier *in vitro* studies [10] used media conditioned by stimulated mast cells. Since the cells, and consequently any proteases trapped by the cells, were removed from this conditioned media, the ratio of HRP-degrading to HRP-generating proteases (e.g. CPA:chymase) could have been greater than it was in the present experiments when the stimulated mast cells were present during the generation of HRP.

When HRP was first identified, we suggested that it might participate in the inflammatory response and thus would be present at sites of inflammation [9]. Our results here with the acute cutaneous inflammatory response in rats (Fig. 4) and with fluids from human arthritic joints and friction blisters (Table 1) support this suggestion. Rheumatoid arthritis is characterized by a chronic state of inflammation of the joints [52]; moreover, synovial fluids and tissues taken from patients with rheumatoid arthritis have increased numbers of mast cells [53] and increased levels of proteases such as cathepsin D and chymase [54], both of which generate HRP [9,10]. The albumin concentration in synovial fluid is approximately 20 mg/mL [55]; twice the concentration used for the *in vitro* generation of HRP [10–12]. While the concentration of HRP we measured in synovial fluids is low (~0.1 nM), this may reflect its generation and rapid degradation by the various proteases present in synovial fluid. Thus, peak concentrations of HRP could be considerably higher. Recent findings by Lee *et al*. [56] have shown that mast cells are essential for the development of inflammatory arthritis in mice. In this study, mast-cell degranulation, as observed in histological sections, was seen before there was clinical evidence of inflammation. This suggests that products released from mast cells contributed to the development of inflammation.

Friction blisters represent an acute mild inflammatory reaction [57,58]. They are produced when frictional forces mechanically cause a separation of epidermal cells at the level of the stratum spinosum. This leads to an accumulation of fluid similar in composition to plasma, but with a lower concentration of albumin (~10 mg/mL, [57,58]). Interestingly, in patients with various skin diseases, the movement of plasma albumin into the fluid of suction blisters is significantly increased compared to that of healthy controls. Moreover, the blister fluids from patients with bullous pemphigoid, epidermolysis bullosa or systemic mastocytosis show significantly elevated levels of histamine and mast-cell protease when compared to plasma levels [59–61]. Thus the conditions are right in these pathologies for the generation of HRP which could then contribute to the inflammation of these disorders.

In conclusion, the results presented here show that the albumin-derived peptide, HRP, has pro-inflammatory actions and is present at sites of acute and chronic inflammation. The localized, rapid generation of peptide signals such as HRP by proteases secreted from the participating cells of inflammation could serve to amplify and perpetuate the inflammatory response. Protease inhibitors or antagonists that block the action of peptides like HRP may therefore be useful in breaking the cycle of inflammation.

Acknowledgments

Lisa Calascibetta and Iris Kaddis contributed to this work as part of their undergraduate training in biology at Tufts University. Terrence Joyce contributed as a summer research fellow at UMass Medical School. We are grateful to Dr. Robert A. Yood of the Division of Rheumatology of the Fallon Clinic and St. Vincent's Hospital in Worcester, MA. for providing the synovial fluid and blood samples from patients with rheumatoid arthritis and Mark J. Doughtie, LATC, CSSC, Director of Sports Medicine at

Tufts University for providing the friction blister fluid samples. We thank Dr. Barry K. Wershil for his help with the experiments on mouse-ear edema, William Boucher and Richard Noring for their outstanding technical assistance and Eileen Magnant for her expert secretarial support. Supported by the Tufts Faculty Research Awards Committee and the National Institutes of Health (AI 19736).

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