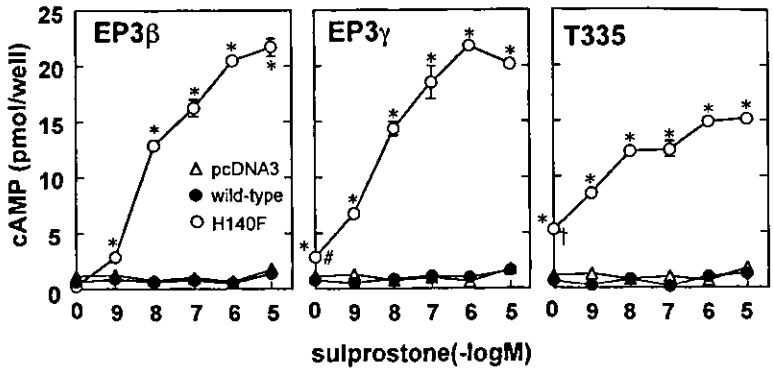

FIG. 7. **Effects of H140F mutation on basal and sulprostone-induced cAMP-producing activity in EP3 receptor isoforms with different C-terminal tails.** The H140F mutation was introduced into three kinds of EP3 receptor forms, EP3β, EP3γ, or T335. HEK293 cells were transfected with each EP3 cDNA or the corresponding mutant receptor cDNA. The cells (2×10^5 cells/well) were treated with the indicated concentrations of sulprostone. The cAMP contents were determined as described under "Experimental Procedures." The results shown are the means ± S.E. of triplicate determinations. *, $p < 0.005$ *versus* each wild-type EP3 form; #, $p < 0.005$ *versus* EP3β-H140F; †, $p < 0.005$ *versus* EP3γ-H140F.

acid residues at the position corresponding to Tyr^{143} of mEP2 in G protein coupling has been pointed out in studies on several kinds of rhodopsin-type receptors (26–29). This site is located at the C-terminal end of a highly conserved i2 loop motif with the following most common sequence: DRY*XX*(V or I)*XX*PL, where *X* is any amino acid (2). The last residue in the consensus sequence, Leu, is replaced with Phe or Met in some members of G_s-coupled receptors. According to the report by Moro *et al.* (27), Leu^{131} in the human M1 muscarinic receptor, which is equivalent to Tyr^{143} of the mEP2 receptor, is critical for stimulation of phosphatidylinositol turnover (G_q coupling). Moreover, they showed that introduction of the equivalent point mutation F139A into the β_2 adrenoreceptor caused a significant loss in isoproterenol-induced cAMP accumulation (G_s coupling). Based on these findings, they concluded that the bulky hydrophobic amino acid at this position is an important amino acid that governs general coupling with any kind of G protein. However, the current finding that conversion of His^{140} into Phe in the EP3 receptor failed to alter G_i coupling (Fig. 6) may suggest that EP3 does not require the particular amino acid at this position for efficient G_i coupling. Indeed, the importance of the hydrophobic amino acid at the corresponding position has not been reported for G_i-coupled receptors. However, the His residue is completely conserved in EP3 receptors derived from various species and is quite unique to EP3 in the GPCR family (Fig. 4). Interestingly, the His residue observed at the key position of EP3 also has a positively charged imidazole structure, which is ineffective in G_s coupling. This can also be interpreted to signify that the His residue participates in the G_i selectivity of EP3 receptor by preventing efficient G_s coupling. In this respect, G_i-coupled receptors contain a nonhydrophobic amino acid at this position; the EDG2 and EDG3 receptors have a basic amino acid, and chemokine receptors, CXCR4 and CXCR6, have a threonine residue (Fig. 4). The variety of amino acids at this position in G_i-coupled receptors may reflect a variety in the way to exert their G_i selectivity and the existence of some other domains such as the C-terminal region of the i3 loop to be required for G_i activation with high efficiency as suggested for the M2 muscarinic receptor (30, 31).

The current study indicated that both EP2 and EP3 require one of the following amino acid residues: Phe, Tyr, Trp, or Leu at position 143 and 140, respectively, for efficient G_s coupling. However, the identity of the side chain moiety (Phe, Tyr, Trp, or Leu) affected different parameters of G_s coupling between EP2 and EP3. In the EP2 receptor, the identity of aromatic moiety seems to affect the EC_{50} values of cAMP production only slightly, suggesting that G_s coupling of the EP2 is also governed by other domains such as the i3 loop, as suggested by previous studies (32). In contrast, in the EP3 receptor, the identity of the aromatic moiety affected the maximal cAMP response without great changes in EC_{50} values. Thus, it seems that the G_s activation efficiency by EP3 completely depends on the side chain moiety at this position, indicating an absolutely pivotal role of this amino acid in G_s coupling of EP3. However, we cannot entirely exclude the possibility that the amino acid identity may alter the G_i activation efficiency, which is usually included in outcomes in a detection system for G_s activity.

Previously, we reported that three C-terminal variants, EP3α, EP3β, and EP3γ, and C-terminal truncated T335 differ in their agonist-dependent G_s activity (EP3γ > T335 > EP3α > EP3β = 0) (21). Since these variants are different only in C-terminal sequence, we speculated that the C-terminal tail may function as a key regulator of G_s coupling of EP3 receptor; β-tail prevents and γ-tail allows the interaction of G_s with the common structure of the EP3 receptor. However, the current study demonstrated that "G_s-excitable" EP3γ further acquired drastic G_s activity, and such gain of function by the H140F mutation is reproduced in C-terminally truncated T335 (Fig. 7). Thus, the gain of G_s activity is independent of C-terminal structure. In our previous report, the G_s activity elicited by EP3γ observed in CHO cells requires agonist concentrations of more than 10^{-6} M, and its maximal response is still not as high as that observed for EP2 or EP4 receptors, and thus the activity is considered to be less efficient. Indeed, the agonist-dependent G_s activity of wild-type EP3γ was undetectable in the current expression system. In contrast, the acquired G_s activity in the mutant receptor is comparable to EP2 and EP4 in terms of the degree of maximal activity and agonist dose dependence and is thought to be essentially different from intrinsic G_s activity appearing in EP3γ. However, the common EP3 structure that allows intrinsic G_s activity may serve as a premise factor for point mutation resulting in gain of G_s coupling with high efficiency. Whether an introduction of a cluster of aromatic residues at the center of i2 loop enables other G_i-coupled receptors to gain G_s coupling is an interesting issue to be examined. We also previously reported that EP3 variants are different also in their constitutive G_i activity (T335 > EP3γ > EP3α > EP3β = 0) (18, 21). It is quite interesting that H140F mutants exhibited basal G_s activity with rank order of T335 > EP3γ > EP3β = 0, which is in good accordance with the potency order of constitutive G_i activity in EP3 variants. Moreover, it should be noted that the agonist-dependent G_s activity in the mutant T335 appeared less than that in the mutant EP3γ; the G_i activity elicited by T335 receptor has been shown to be completely constitutive. These results suggested that the effects of i2 loop mutation on G_s coupling of EP3 is independent of C-terminal structure, which is likely to govern the balance of constitutive and agonist-induced G protein activation as observed in the G_i activity of the EP3 isoforms. Importantly, these results suggest a general role for the C-terminal tail in G protein coupling; the

C-terminal tail plays a critical role in constraining the constitutive activity irrespective of class of coupling G proteins.

One of the remarkable findings in this study is that a cluster of aromatic amino acids beginning with Tyr^{143} or the corresponding residue is required for G_s coupling with high efficiency in prostanoid receptors (Fig. 5). This feature, the existence of three bulky aromatic amino acids following the conserved proline residue, is unique to G_s-coupled prostanoid receptors (Fig. 4). The 3 amino acids just after the proline in the four G_s-coupled prostanoid receptors are YFY, FFY, or YLY, whereas the other members contain HWY, LIH, IFH, or FSR. The present study demonstrated that the existence of an uncharged aromatic residue at the first position is the most critical for G_s coupling. However, the simultaneous introduction of alanine mutations at the following two residues resulted in a significant loss of efficiency in G_s activity in EP2 and EP3-H140Y receptors. Moreover, the existence of an aromatic residue (Phe^{144} in EP2 and Trp^{141} in EP3) at the second position appears to be required for G_s coupling with high efficiency. In contrast, the Tyr residue at the third position is dispensable if the first two residues are aromatic, but this residue is likely to take part in G_s coupling in the absence of an aromatic residue at the second position. Based on these results, we concluded that G_s coupling is controlled by the three aromatic amino acids following the conserved Pro residue with a rank order of contribution of first > second > third residue in the prostanoid receptors.

How does the aromatic residue contribute to G_s coupling with high efficiency? The rank order of amino acids critical for efficient G_s coupling of EP2 and EP3 receptors is as follows; Phe > Tyr > Trp > Leu ≫ other amino acids = 0 (Figs. 2 and 3). There is no doubt that the C-terminal 5 amino acids of the $G\alpha$ subunit are important for its selective binding to the receptors; both G_s and $G_{q/11}$ families contain a Tyr residue at −4 from the C-terminal end, whereas the G_i family contains a quite different amino acid, cysteine, at this position (33–35). Recently, Liu *et al.* demonstrated that the aromatic moiety of the Tyr residue conserved at −4 from the C-terminal end of the $G\alpha_s$ and $G\alpha_q$ plays a key role in receptor/G protein interactions with high efficiency (36). By point mutation analysis, they demonstrated that agonist-induced $G\alpha_{11}$ activation is controlled by the identity of the −4 residue with the rank order of Phe > Tyr > Trp ≫ other amino acids. Although they did not examine the effect of the Leu mutation, the three most effective amino acids, Phe, Tyr, and Trp are completely identical to the amino acids critical for G_s coupling at the key position in both EP2 and EP3 receptors. From these results, we speculate that the bulky aromatic amino acid in the i2 loop takes part in recognition of the Tyr residue conserved in G_s and G_q through a mechanism such as π electron interactions. In such case, a cluster of aromatic residues may contribute to strengthen the interaction with or to accelerate the recognition of the tyrosine residue at the −4-position of G_s. Recently, Erlenbach *et al.* (29) employed an yeast screening system, in which random mutations were introduced into the G_s-coupled vasopressin V2 receptor, to detect amino acid mutations affecting receptor interaction with the C-terminal tail of G proteins. They found that a single amino acid substitution at Met^{145} into Leu or Trp within the i2 loop equivalent to Tyr^{143} of mEP2 allowed the V2 receptor to couple to both G_q and G_s (29). They also discussed the possibility that Met^{145} is a strong candidate site for interaction with G proteins based on the analogy of the high resolution x-ray structure of bovine rhodopsin. According to the original report, the i2 loop exhibits an L-like structure when viewed parallel to the membrane plane but lacks regular secondary structure (37). Because the cytoplasmic extension of TM III and the N-terminal segment of the i2 loop show considerable sequence homology among GPCRs of the rhodopsin family, it is likely that the i2 loop of the EP2 receptor adopts a structure similar to that observed in rhodopsin. If this is correct, the cluster of aromatic residues from Tyr^{143} to Tyr^{145} is predicted to be located just N-terminal of the bend of the L-like structure that is a characteristic feature of the i2 loop where it is easily accessible for interactions with G proteins. Taken together, we propose a cluster of aromatic amino acids in the i2 loop as a strong candidate for an interaction site with the G_s protein.

The current study demonstrated that interchanging of the i2 loop or the N-terminal or C-terminal half region of the i2 loop between EP2 and EP3 left the individual binding affinities and the specificity and expression levels of the receptors unaffected. These results may reflect the fact that the i2 loops do not directly contribute to the formation of the ligand binding pocket. On the contrary, the interchanging of the i1 loops resulted in loss of binding ability of both receptors. Although all EP receptors can recognize PGE_2 as a natural ligand, it has long been suggested that each EP receptor recognizes different functional groups of agonists (38). Since it was recently proposed that both TM I and II contribute to receptor recognition of different functional groups of prostanoid ligands (39), the i1 loop of the prostanoid receptors may be critical in the formation of subtype-specific ligand binding pockets. Interchanging of the i3 loops differently affected the binding properties of the wild type receptor; EP2 lost but EP3 retained the ability to bind to PGE_2. This finding may reflect the fact that EP2 requires an i3 loop of appropriate length to form a binding pocket that can hold prostaglandin derivatives with a bulky structure (25).

In summary, we have demonstrated that a cluster of aromatic amino acids at the center of the i2 loop plays a key role in G_s coupling, at least in the prostanoid receptors. This study will be of help to understand the molecular mechanisms of G protein coupling selectivity by the individual GPCRs.

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Expression of L-histidine decarboxylase in granules of elicited mouse polymorphonuclear leukocytes

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Infiltrating polymorphonuclear leukocytes (PMN) in the peritoneal cavity were found to express L-histidine decarboxylase (HDC), the rate-limiting enzyme of histamine synthesis, in a csein-induced peritonitis model. Expression of HDC was detected in the elicited PMN, but not in the peripheral blood leukocytes. The peritoneal lavage fluids in this model were found to augment histamine synthesis in PMN isolated from the bone marrow. Rapid post-translational processing of HDC was observed in PMN, and the dominant form of HDC was the mature 53-kDa form, which was found to co-localize with a granule enzyme, matrix metalloproteinase-9 (MMP-9). Treatment of PMN with the phorbol ester PMA, which stimulates the release of MMP-9, did not liberate the granular HDC. Immunofluorescence studies using an anti-HDC antibody strongly suggested that HDC is bound to the cytosolic side of the granule membranes. These observations suggest that HDC is induced upon infiltration of PMN into the mouse peritoneal cavity and that histamine is synthesized by HDC attached to the granule membranes of PMN.

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1 Introduction

Histamine has been reported to play various roles in the modulation of immune and inflammatory responses via the H_1 and H_2 receptors [1], such as increase in vascular permeability [2], leukocyte rolling [3], cytokine production [4, 5], polarization of dendritic cells [6], and T cell and antibody responses [7, 8]. These studies suggest that endogenous histamine production can modulate immune responses, although the exact source of this histamine remains to be fully determined. Many of these studies have assumed that the histamine released from mast cells and basophils via degranulation is important in such kinds of immune regulation. However, since this histamine release is generally observed at the onset of inflammatory responses, it is unlikely that only histamine released from mast cells and basophils can modulate the immune responses, especially during the late phase responses. In an air pouch-type allergic inflammation model in rats, the release of histamine into the pouch fluid was found to be biphasic [9]: in the anaphylactic phase, activated mast cells in the pouch released histamine, causing a transient increase in vascular permeability via the H_1 receptors [2], and in the following chronic phase, cells of unknown identity, which were not mast cells, produced histamine, causing the inhibition of leukocyte infiltration into the pouch via the H_2 receptors [10]. During the chronic phase of inflammation, an intensive infiltration of polymorphonuclear leukocytes (PMN) and macrophages is usually observed. A detectable amount of histamine synthesis was found to be induced in the murine myelomonocytic cell line WEHI-3B, in parallel with their differentiation into macrophages [11]. Mirossay et al. have demonstrated the existence of trace amounts of L-histidine decarboxylase (HDC) activity and histamine content in macrophages as well as in the human promyelocytic leukemia cell line HL-60, which can be differentiated into granulocytes by all-*trans*-retinoic acid treatment [12]. These *in vitro* studies suggest that both PMN and macrophages may be involved in histamine synthesis during the chronic phase of the inflammatory response. Although it still remains unknown as to which cell type is responsible for histamine synthesis *in vivo*, it is important to clarify the regulation of histamine synthesis by these cell types in order to appreciate the modulatory role of histamine in immune and inflammatory responses.

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Abbreviations: BM-PMN: PMN precursors from bone marrow **ER:** Endoplasmic reticulum **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase **HDC:** L-Histidine decarboxylase **MMP-9:** Matrix metalloproteinase-9 **PDI:** Protein disulfide isomerase **SLO:** streptolysin-O

We previously described the post-translational processing and intracellular localization of HDC (EC 4.1.1.22), the rate-limiting enzyme in histamine synthesis in mammals, in the rat basophilic/mast cell line RBL-2H3 [13]. HDC was found to be translated as a 74-kDa precursor form in the cytosol, which is then processed into its mature 53-kDa form. In addition, histamine synthesis was detected in two distinct compartments, in the cytosol and in granules. On the other hand, in the preliminary study, we have found that PMN are the dominant cell type responsible for histamine synthesis in an experimental casein-induced peritonitis model [14]. Granules in human PMN have been classified into three types; azurophil/primary, specific/secondary, and gelatinase/tertiary granules [15], whereas such a classification of neutrophil granules remains to be fully determined in mice. Although the previous observation that azurophil granules in PMN share several characteristics with histamine-containing granules in mast cells [16] indicates the granular localization of HDC in PMN, the exact intracellular localization of HDC in PMN remains to be clarified. In this study, we investigated the post-translational processing and intracellular localization of HDC to elucidate the regulation of histamine synthesis in PMN.

2 Results

2.1 Expression of HDC in PMN

In our preliminary study, we have found that PMN are responsible for histamine synthesis in an experimental casein-induced peritonitis model [14]. In this model, a drastic and transient increase in HDC enzyme activity in total peritoneal cells was observed, reaching a peak level 5 h after casein injection, at which time an intensive infiltration of PMN into the peritoneal cavity was observed ([14] and data not shown). PMN were purified from the peritoneal cells 5 h after casein injection. Immunofluorescence studies revealed that the purified PMN fraction (>98% purity, confirmed by May-Grünwald-Giemsa staining) was immunoreactive to both an anti-HDC and an anti-CD11b antibody (Fig. 1A). The immunofluorescence pattern indicated a granule-like localization of HDC in the PMN. On the other hand, HDC^+ cells were not found in total leukocytes from peripheral blood, both in control mice and in mice injected with casein, although a significant number of $CD11b^+$ cells were observed in peripheral blood leukocytes from both mice (Fig. 1B). In addition, no detectable amount of HDC activity could be measured in the homogenates of peripheral blood leukocytes collected from mice treated with or without casein for 5 h (data not shown). These results indicate that HDC may be induced in response to inflammatory stimuli in

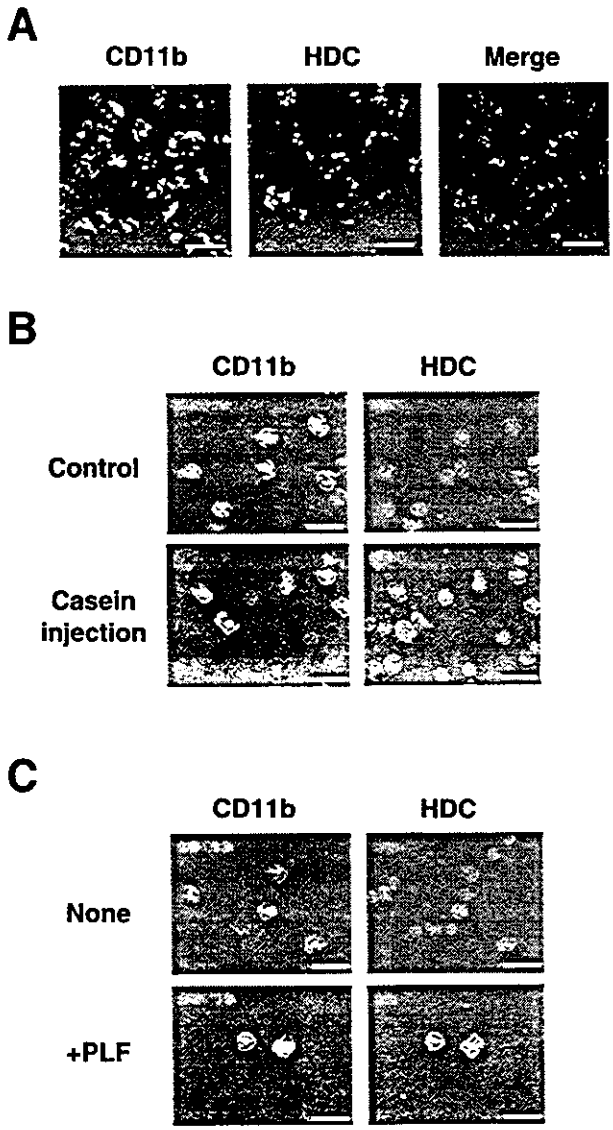

Fig. 1. Expression of HDC in infiltrated PMN, and not in peripheral blood leukocytes, in the casein-induced peritonitis model. (A) PMN were purified from peritoneal cells 5 h after casein injection. Cells were centrifuged onto slide glasses and immunofluorescence studies were performed with an anti-HDC antibody (1:500) followed by incubation with an Alexa594-conjugated anti-rabbit IgG antibody (1:1,000; HDC) and a FITC-conjugated anti-CD11b antibody (1:1,000; CD11b). Bars =20 μm. (B) Peripheral blood was collected from control mice and mice 5 h after casein injection, and the leukocyte fraction was purified. HDC-immunoreactive cells were never observed in any of the multiple microscopic fields analyzed. Bars =20 μm. (C) Peripheral blood leukocytes were collected from mice 5 h after casein injection. Total leukocytes were incubated for 6 h under standard culture conditions in the presence (PLF) or absence (None) of the peritoneal lavage fluid obtained 5 h after the injection. Bars =20 μm.

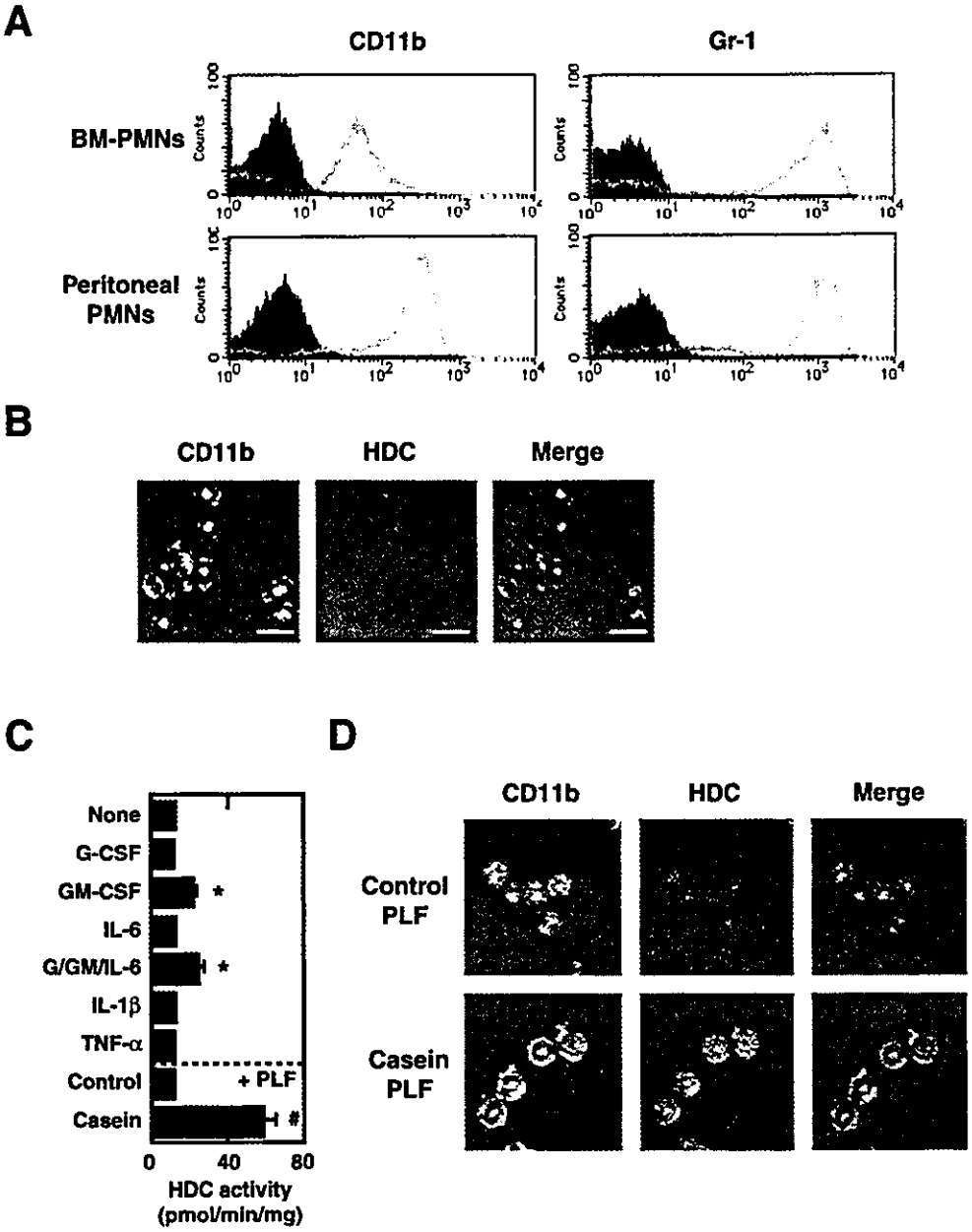

Fig. 2. Induction of HDC in BM-PMN. (A) PMN were isolated from bone marrow by density gradient fractionation (BM-PMN). The lineage of the cells was confirmed by flow cytometry with an anti-CD11b antibody and an anti-Gr-1 antibody. The closed area profiles were obtained with the isotype control antibody. (B) BM-PMN were subjected to immunofluorescence study as described in the legend to Fig. 1. Bars =20 μm. (C) BM-PMN were incubated for 3 h in the absence (None) or presence of the indicated cytokines (20 ng/ml), the peritoneal lavage fluids obtained from mice injected with casein (Casein PLF) or those from control mice (Control PLF). Cells were harvested and HDC activity was measured. Values are represented as the means ± SEM (n=3–6). *$p<0.05$ is regarded as significant by the Student's t test (*vs.* None). #$p<0.05$ is regarded as significant by the Student's t test (*vs.* Control PLF). (D) BM-PMN were incubated for 3 h in the presence of the peritoneal lavage fluids obtained from mice injected with casein (Casein PLF) or from control mice (Control PLF). Incubated BM-PMN were subjected to immunofluorescence study as described in the legend to Fig. 1. Bars =20 μm.

the cavity. To confirm this hypothesis, we collected peripheral blood leukocytes and peritoneal lavage fluid from peritoneal cavities 5 h after casein injection. After incubation of the peripheral blood leukocytes with the lavage fluid for 6 h at 37°C, a significant number of HDC$^+$ cells could be detected (Fig. 1C).

2.2 Induction of HDC in PMN isolated from bone marrow

Since a method for purification of PMN from murine peripheral blood leukocytes remains to be established, we isolated PMN precursors from the bone marrow (BM-PMN) by density gradient fractionation. Flow cytometric analysis demonstrated that greater than 80% of this fraction was immunoreactive to both an anti-CD11b and an anti-Gr-1 antibody (Fig. 2A). In comparison with casein-induced peritoneal PMN, similar levels of surface expression of Gr-1 and lower levels of CD11b were observed in BM-PMN. Immunofluorescence studies revealed that $CD11b^+$ cells in BM-PMN were not immunoreactive to the anti-HDC antibody (Fig. 2B). We then investigated effects of peritoneal lavage fluids and several cytokines on the induction of histamine synthesis in BM-PMN. A drastic increase in HDC activity was observed in BM-PMN incubated for 3 h in the presence of peritoneal lavage fluids collected from mice injected with casein, but not in the presence of fluids collected from control mice (Fig. 2C). Induction of HDC by the lavage fluids was also confirmed by immunofluorescence study with the anti-HDC antibody (Fig. 2D). Among the cytokines tested, treatment with GM-CSF was found to slightly but significantly augment HDC activity in BM-PMN (Fig. 2C). IL-1β, IL-6, TNF-α, and G-CSF did not induce HDC activity in BM-PMN.

2.3 Expression and enzymatic activity of HDC in PMN

We then investigated the expression of mRNA and protein and the enzymatic activity of HDC in peritoneal PMN. Northern blot analyses demonstrated a high level of HDC mRNA expression in PMN immediately after purification, followed by a rapid decrease under culture conditions (Fig. 3A). Immunoblot analysis using the anti-HDC antibody revealed that the dominant molecular species in peritoneal PMN is the 53-kDa form (Fig. 3B). Purified PMN showed a significant level of HDC activity (~80 pmol/min/mg protein), which gradually decreased under standard culture conditions for up to 6 h (Fig. 3C). Post-translational processing of HDC was confirmed by pulse-chase experiments (Fig. 3D, E). The 74-kDa precursor form was found to be completely converted into its mature 53-kDa form within 1 h. A band of approximately 120 kDa with strong labeling was detected, but was still detectable in the presence of excess amounts of the antigen, glutathione-S-transferase HDC fusion protein (data not shown), and hence we conclude that this band is from an unrelated protein detected in our system.

2.4 Co-localization of HDC with MMP-9

We investigated the intracellular localization of HDC. PMN were double-stained with the anti-HDC antibody and an antibody against matrix metalloproteinase-9 (MMP-9, gelatinase B), which is known to be a granule proteinase in PMN. In confocal microscopic observations, HDC and MMP-9 were found to co-localize in the granules of PMN (Fig. 4A), indicating that HDC is localized in the MMP-9-containing granules of PMN.

2.5 Unchanged localization of HDC upon stimulation with PMA

Since previous studies have demonstrated that MMP-9 is liberated from PMN upon treatment with phorbol 12-myristate 13-acetate (PMA) [17], extracellular release of HDC was investigated. A dose-dependent release of MMP-9 was measured as both enzyme activity and immunoreactive protein upon treatment of PMN with PMA for 30 min (Fig. 4B), whereas no release of HDC enzyme activity (data not shown) and immunoreactive protein was detected under the same conditions (Fig. 4B). The absence of MMP-9 and the granule-associated expression of HDC upon PMA treatment were also confirmed by immunofluorescence studies (Fig. 4C). Although relatively high levels of spontaneous histamine release (approximately 20%) were detected, a significant amount of stimulated release was detected upon treatment with PMA (Table 1). On the other hand, neither *N*-formyl-methionyl-leucyl-phenylalanine (up to 10 μM) nor leukotriene B_4 (up to 1 μM) was found to induce a significant histamine release (data not shown).

2.6 Membrane orientation of HDC

We then investigated in detail the localization of HDC in the granules of PMN using streptolysin-O (SLO), which selectively permeabilizes plasma membranes [18]. Neither HDC activity nor a band immunoreactive to the anti-HDC antibody was detected in the cytosol fractions obtained after SLO treatment, whereas more than 80% of lactate dehydrogenase activity was recovered (data not shown), excluding the possibility that HDC is a cytosol-soluble protein. Immunoreactive signals were observed with the anti-HDC antibody, and not with the anti-MMP-9 antibody, in the SLO-treated PMN (Fig. 5), indicating that HDC was accessible to the antibody entering from the cytosolic side whereas MMP-9 was not. When the SLO-treated cells, after fixation, were further permeabilized with 1% Triton X-100, co-localization of HDC and MMP-9 was again confirmed.

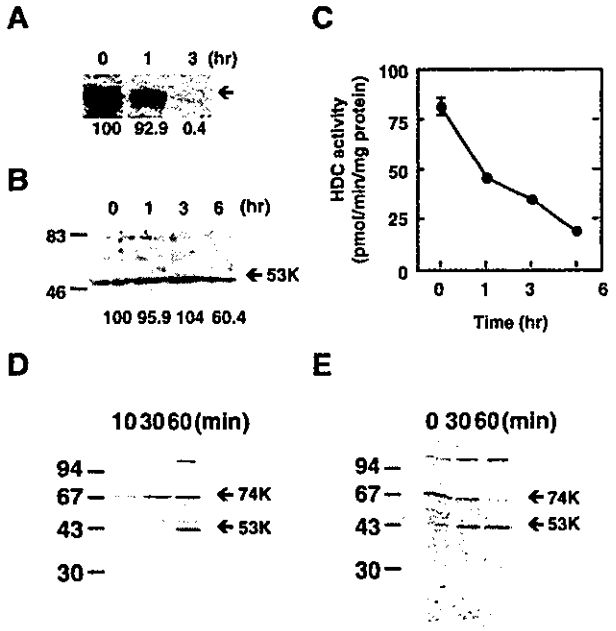

Fig. 3. Expression and post-translational processing of HDC in peritoneal PMN under standard culture conditions. Peritoneal PMN were incubated under standard culture conditions for the indicated periods. (A) Northern blot analyses were performed using a ^{32}P-labeled probe for HDC (total RNA, 3 μg/lane). The relative intensity of each band was normalized using the probe for GAPDH and is presented below the panel.(B) Immunoblot analysis was performed using the anti-HDC antibody (1:500). The arrow indicates the 53-kDa mature form of HDC (53 K). The relative intensity of each band was measured densitometrically and is presented below the panel. The 74-kDa form of HDC was not detectable. (C) Enzyme activity of HDC was measured and presented as mean ± SEM (n=5). (D) PMN (2×10^7 cells/lane) were metabolically labeled with [^{35}S]methionine in methionine-free medium for the indicated periods. Cells were immunoprecipitated with the anti-HDC antibody. The immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. (E) PMN labeled with [^{35}S]methionine for 30 min were subjected to chase experiments in the presence of 1 mM methionine for the indicated periods. The arrows in (D) and (E) indicate the precursor form of HDC (74 K) and the mature form of HDC (53 K).

3 Discussion

In the previous report [14] and in the current study we demonstrated that isolated PMN express HDC and produce histamine in a casein-induced experimental peritonitis model. Expression of HDC in purified peritoneal PMN was demonstrated at both mRNA and protein levels. These results are consistent with previous findings by Shiraishi et al. [19] that infiltrating leukocytes in an air pouch-type allergic inflammation model and in a casein-induced peritonitis model in rats expressed HDC mRNA.

Table 1. Histamine release from PMN stimulated with PMA[a)]

PMA (nM)	Total (ng/10^7 cells)	Release (ng/10^7 cells)	Net release (%)
0	275±15.4	54.2±13.9	
10	272±39.7	79.8±4.41	8.58±5.40
30	278±25.8	83.9±14.8	10.1±4.66
100	287±40.5	96.9±19.6	13.5±7.52

[a)] PMN purified from peritoneal cells 5 h after casein injection were incubated under standard culture conditions in the presence of the indicated concentrations of PMA for 30 min. Culture medium and cells were separated by centrifugation at 2,000×g for 5 min at 4°C. Values for net release were calculated by subtracting the value for spontaneous release (PMA 0 nM; 54.2±13.9 ng/10^7 cells). The values are presented as means ± SEM (n=5).

Although their results strongly suggest that PMN are the dominant source of *de novo* synthesis of histamine (~1.2 pmol/min/mg protein in infiltrating cells), they did not exclude the possibility that macrophages may produce some of this histamine. We have determined the specific activity of HDC in the peritoneal PMN (~80 pmol/min/mg protein, Fig. 3A), which was much higher than previously reported for activated macrophages and mast cells (<10 pmol/min/mg protein) [10, 20]. Furthermore, it is notable that HDC expression was induced in PMN in the peritoneal cavity, since peripheral blood leukocytes did not express HDC. It has been reported that CD11b expression in intravascular neutrophils can be enhanced by the addition of peritoneal fluid supernatant in a glycogen-induced peritonitis model [21]. In the casein-induced model, we have also found that peritoneal lavage fluid has the potential to induce HDC protein in peripheral blood CD11b$^+$ cells and in PMN isolated from the bone marrow (BM-PMN). Therefore, some humoral factors may exist in the peritoneal cavity that can induce HDC expression in PMN. The rapid decrease of HDC mRNA expression under culture conditions supports this hypothesis. We found an increase in HDC activity in BM-PMN incubated in the presence of GM-CSF. G-CSF and IL-6 demonstrated neither additive nor synergistic effects on GM-CSF-mediated induction of HDC, although these cytokines are also involved in regulation of PMN functions. Since the potential of the peritoneal lavage fluids to augment histamine synthesis was much greater than that of GM-CSF, it is possible that the peritoneal lavage fluids contain other inducing factors for HDC. TNF-α is one of the possible candidates, since Endo has previously reported that a systemic injection of TNF-α was able to induce histamine synthesis in mouse bone marrow cells and

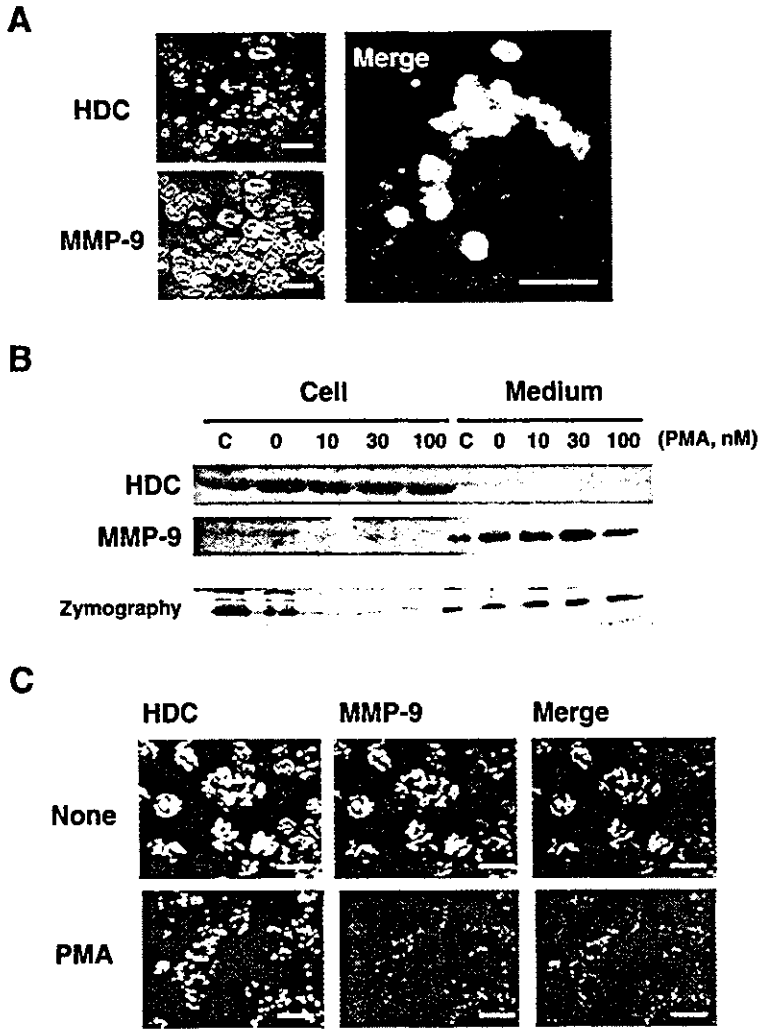

Fig. 4. Unchanged localization of HDC in granules upon treatment with PMA. (A) Peritoneal PMN were centrifuged onto slide glasses. Immunofluorescence studies were performed with an anti-HDC antibody (1:500, HDC) and an anti-MMP-9 antibody (1:500, MMP-9) followed by incubation with a FITC-conjugated anti-rabbit IgG antibody (1:100, green) and a rhodamine-conjugated anti-goat IgG antibody (1:100, red). Bars =20 μm. A magnified view is presented (Merge, right panel). Bar =5 μm. (B) PMN were incubated under standard culture conditions in the presence of the indicated concentrations of PMA for 30 min. Control experiments (C) were performed without incubation. Culture medium (Medium) and cells (Cell) were separated by centrifugation at 2,000×*g* for 5 min at 4°C. Each fraction was subjected to immunoblot analyses using the anti-HDC antibody (1:500) and the anti-MMP-9 antibody (1:500). Gelatin zymography was performed as described in section 4.12. (C) PMN were incubated under standard culture conditions in the presence (PMA) or absence (None) of 100 nM PMA for 30 min. Immunofluorescence studies were performed as described above. Bars =10 μm.

spleen cells [22] and since a significant amount of TNF-α release from mast cells has been detected during the anaphylactic phase of a mouse peritonitis model [23]. However, TNF-α may not be involved in the induction of HDC in our model, as TNF-α did not augment HDC activity in BM-PMN and an intraperitoneal injection of an anti-TNF-α neutralizing antibody did not inhibit the induction of HDC (data not shown). Another proinflammatory cytokine, IL-1β, was also found to have no inducible effects on HDC expression in PMN. Further studies are surely required to determine the factors responsible for induction of HDC.

The enzymatic activity of HDC in peritoneal PMN was found to rapidly decrease under culture conditions, before the decrease in HDC mRNA and protein. This result indicates the occurrence of enzymatical inactivation of HDC in peritoneal PMN in addition to degradation of its mRNA and protein, although such inactivation has not been previously reported. Rapid suppression of his-

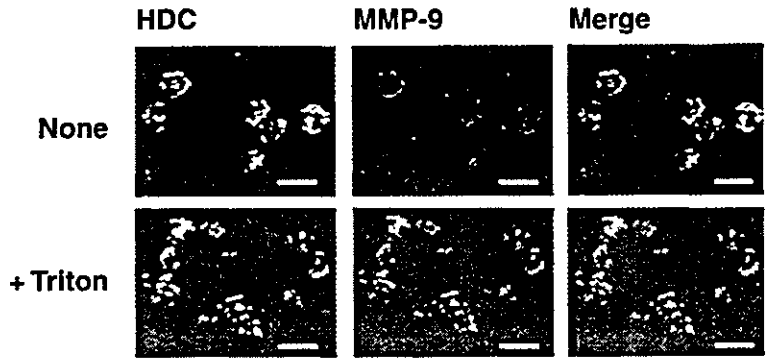

Fig. 5. Granular membrane orientation of HDC. PMN were treated with SLO (12,500 U/ml) and fixed. Cells were further permeabilized with (+ Triton) or without (None) 1% Triton X-100 and then stained using the anti-HDC antibody or the anti-MMP-9 antibody as described in the legend to Fig. 3. Bars =10 μm.

tamine synthesis in peritoneal PMN may contribute to the prevention of exacerbated inflammatory responses, which could be induced by prolonged histamine release.

The immunofluorescence studies using the anti-HDC antibody revealed a granular localization of HDC in PMN. We previously demonstrated that HDC was translated in the cytosol and targeted to the endoplasmatic reticulum (ER), where post-translational processing occurred in the rat mast cell line RBL-2H3 [13]. We also observed that histamine was produced in both the cytosol and the granule fractions of the cells, mediated by the 74-kDa form and the 53-kDa form, respectively. Compared with the intracellular localization of HDC in the mast cell line, which is mainly in the ER, the current study demonstrated that HDC is co-localized with MMP-9 in PMN, indicating the granular localization of HDC. These results indicate that HDC may be efficiently processed into the mature 53-kDa form and transported to the gelatinase-containing granules in PMN. Indeed, the pulse-chase experiments showed the rapid and complete processing of HDC as only the 53-kDa form was detected upon immunoblot analyses using the anti-HDC antibody.

We previously demonstrated that the 53-kDa form of HDC was not accessible to the anti-HDC antibody in SLO-treated RBL-2H3 cells [13], which strongly suggests that the 53-kDa form of HDC is localized in the luminal compartments of the cells. Indeed, the 53-kDa form was found to be resistant to trypsin digestion in SLO-treated RBL-2H3 cells. On the contrary, the 53-kDa form was accessible to the anti-HDC antibody in SLO-treated PMN. Since HDC was not released upon SLO-treatment, HDC may be bound to the cytosolic side of granule membranes in PMN. We have no experimental evidence giving a clear explanation for this inconsistency in the localization of HDC between mast cells and PMN. Membrane orientation and targeting of HDC remains largely unknown. Using the *in vitro* translation system with rabbit reticulocyte lysates, we previously demonstrated that targeting of the 74-kDa form to the microsomal membranes occurs post-translationally [24], although the exact mechanism remains to be determined. Since the 74-kDa form of HDC lacks the amino-terminal signal sequence, which can bind to the signal recognition particle, the membrane targeting of HDC may be mediated by an unknown mechanism. Further analyses are required to clarify this mechanism.

Compared with mast cells, histamine release from PMN is characterized by high levels of spontaneous release, indicating the rapid turnover of nascent histamine in PMN. On the other hand, the observation that relatively small amounts of histamine were released upon PMA treatment indicates the existence of heterogeneous pools of granule histamine. Granule-associated localization of HDC may contribute to uptake of nascent histamine into granules, and spontaneous release of granule contents may occur in PMN infiltrated into the peritoneal cavity. The transport system for histamine across the plasma membrane and vesicular membranes remains largely unknown. There has been no experimental evidence that excludes the possibility that histamine produced in the cytosol may be directly released via a transporter in the plasma membrane. Indeed, we have recently reported that histamine is transported into the cytosol of mouse macrophages in a Na^+-independent manner and that cytosolic histamine is liberated from the cells after depletion of extracellular histamine, although the molecular identity of the transporter remains to be determined [25]. We surely need further experimental evidence to address this problem.

It has been demonstrated that large amounts of histamine are transiently released from mast cell granules in the anaphylactic phase of inflammatory responses,

whereas continuous histamine release is usually observed in the late phase, which is accompanied by an increase in histamine synthesis [9]. These distinct characteristics of histamine release may reflect the difference in the fashion of histamine synthesis and storage between mast cells and PMN; histamine is accumulated and stored in the granules of mast cells, whereas it is rapidly released from PMN.

In summary, we demonstrated in this study that HDC is induced in PMN infiltrated into the peritoneal cavity and that the dominant 53-kDa form, which is converted by a rapid post-translational processing, is localized on the cytosolic side of gelatinase-containing granules in PMN. PMN may be one of the important sources of histamine in inflammatory responses.

4 Materials and methods

4.1 Materials

An anti-HDC antibody was prepared as described [26]. This antibody was raised against the partial amino acid sequence (residues 1–210) of mouse HDC and can recognize both the precursor 74-kDa form and the mature 53-kDa form of HDC [13]. The following materials were purchased from the sources indicated: an anti-MMP-9 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); an anti-protein disulfide isomerase (PDI) antibody from StressGen (Victoria, BC); a FITC-conjugated anti-CD11b antibody and a phycoerythrin-conjugated anti-Gr-1 antibody from PharMingen (San Diego, CA); a FITC-conjugated anti-rabbit IgG antibody and a rhodamine-conjugated anti-mouse IgG antibody from Leinco Technologies, Inc. (Ballwin, MO); a rhodamine-conjugated donkey anti-goat IgG antibody and a FITC-conjugated donkey anti-rabbit IgG antibody from CHEMICON (Temecula, CA); an Alexa594-conjugated anti-rabbit IgG antibody from Molecular Probes, Inc. (Eugene, OR); polyvinylidene difluoride (PVDF) membranes from Millipore (Tokyo, Japan); a horseradish peroxidase-conjugated anti-rabbit IgG antibody from Dako (Glostrup, Denmark); ISOGEN from Nippon Gene (Tokyo, Japan); Percoll, ECL Western blot detection reagent and protein A Sepharose CL-4B from Amersham Pharmacia (Uppsala, Sweden); Biodyne A from Pall BioSupport Corporation (East Hills, NY); SLO, *N*-formyl-methionyl-leucyl-phenylalanine, and leukotriene B_4 from Sigma Aldrich (St. Lois, MO); [^{35}S]methionine (1,000 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) from Du Pont-New England Nuclear (Boston, MA). All other chemicals were commercial products of reagent grade.

4.2 Animals

Female BALB/c mice (7–8 weeks old) were obtained from Shimizu Experimental Animal Lab, Co. Ltd. (Kyoto, Japan). All experiments were performed according to the Guideline for Animal Experiments of Kyoto University.

4.3 Isolation of elicited peritoneal PMN

Female BALB/c mice were used for all experiments. Casein in saline (5%, w/v, 2 ml/cavity) was injected intraperitoneally. Peritoneal cell types were determined by microscopic observation after May-Grünwald-Giemsa staining. Cells in the peritoneal cavity were harvested 5 h after injection by lavage of the cavities with 3 ml sterile PBS. Lavage fluids were centrifuged at 200×g for 5 min at 4°C, and the pellet was resuspended in 2 ml PBS. PMN were purified by centrifugation on discontinuous Percoll gradients [27]. Briefly, 2 ml cell suspension were carefully layered on top of the discontinuous Percoll gradient prepared with 2 ml solution B (density 1.090 g/ml) and 2 ml solution A (density 1.070 g/ml). After centrifugation at 500×g for 20 min at 4°C, two distinct leukocytic layers were obtained. The lower band was collected as the PMN fraction and washed twice in PBS. Determination of the cell population by May-Grünwald-Giemsa staining indicated that more than 98% of the cells obtained were neutrophils, whereas the rest were mononuclear cells.

4.4 Isolation of PMN from bone marrow

Bone marrow cells were harvested from the femurs of female BALB/c mice and washed in Hanks' balanced salt solution. PMN in bone marrow were isolated according to the procedure described [28] with minor modifications. Briefly, bone marrow cells were resuspended in PBS (5×10^7–7×10^7 cells/ml) and carefully layered on top of the discontinuous Percoll gradient consisting of three layers [29] (3 ml each; densities 1.082, 1.075, 1.053 g/ml). After centrifugation at 500×g for 30 min at 4°C, the bottom fraction (1 ml) was collected as the PMN fraction (BM-PMN) and washed twice in PBS. Determination of the cell population was performed by May-Grünwald-Giemsa staining and by flow cytometry.

4.5 Culture of peritoneal and bone marrow PMN

Purified PMN or bone marrow PMN were cultured in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated fetal calf serum in a fully humidified 5%-CO_2 atmosphere (standard culture conditions) for the indicated times. The viability of greater than 98% of the cells was confirmed by trypan blue exclusion.

4.6 Flow cytometric analysis

Flow cytometric analyses were performed with a FACSCalibur (Becton Dickinson) using a FITC-conjugated anti-CD11b

antibody and a phycoerythrin-conjugated anti-Gr-1 antibody to determine the neutrophil populations. The population of positive cells was determined by comparison to cells stained with FITC- or phycoerythrin-conjugated isotype-matched immunoglobulin.

4.7 Immunofluorescence studies

PMN were centrifuged onto round cover glasses (■=18.0 mm), which were then placed in 12-well culture plates. Immunofluorescence studies were performed as described [13]. An anti-HDC antibody (1:500), an anti-MMP-9 antibody (1:500), an anti-PDI antibody (1:500) and a FITC-conjugated anti-CD11b antibody (1:1,000) were used as primary antibodies. Alexa594-conjugated goat anti-rabbit IgG antibody (1:1,000) was used for HDC and CD11b double-staining. A rhodamine-conjugated donkey anti-goat IgG antibody (1:100) and a FITC-conjugated donkey anti-rabbit IgG antibody (1:100) were used for MMP-9 and HDC double-staining, respectively. Stained cells were observed by confocal microscopy (MRC-1024, Bio-Rad Laboratories, Hercules, CA).

4.8 Northern blot analyses

Total RNA was extracted from purified PMN using ISOGEN according to the manufacturer's instructions, separated (3 μg/lane) by electrophoresis on a 1.5% agarose/formaldehyde gel and transferred onto a Biodyne A membrane in 20× SSC (1× SSC is composed of 0.15 M NaCl and 0.015 M sodium citrate) by capillary blotting. Hybridization was performed with a ^{32}P-labeled cDNA fragment specific for murine HDC (Pvu II-digested fragment) in hybridizing solution (6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA) at 68°C overnight [30]. The filter was rinsed twice in 2× SSC containing 1% SDS at room temperature and twice in 0.2× SSC containing 0.1% SDS at 60°C. A ^{32}P-labeled probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in rehybridization for the loading control. The filter was then analyzed using a Fujix BAS 2000 Bio-Imaging Analyzer.

4.9 Immunoblot analyses

PMN were homogenized in 50 mM HEPES-NaOH pH 7.3, containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2% polyethylene glycol 300, 0.2 mM PMSF and 0.1% TritonX-100 and centrifuged at 15,000×g for 30 min at 4°C. The resultant supernatant (50 μg/lane) was subjected to SDS-PAGE (10% slab gel), and the separated proteins were transferred electrophoretically to a PVDF membrane. Immunoblot analysis was performed as described [13]. An anti-HDC antibody (1:500) or an anti-MMP-9 antibody (1:500) was used as the primary antibody, and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3,000) or a horseradish peroxidase-conjugated anti-goat IgG antibody (1:3,000) was used as the secondary antibody. The membranes were stained using an ECL kit according to the manufacturer's instructions.

4.10 Histidine decarboxylase assay

PMN were rinsed with PBS followed by centrifugation, and the cell pellet was lysed (1×10^7 cells/ml) with 50 mM HEPES-NaOH pH 7.3 containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2% polyethylene glycol 300, 0.2 mM PMSF and 0.1% Triton X-100 on ice for 30 min. The cells were centrifuged at 100,000×g for 1 h at 4°C, and the supernatant was used for the measurement of histidine decarboxylase activity as described [13]. The precipitate fraction exhibited no detectable enzymatic activity. The histamine formed was separated on a cation exchange column, WCX-1 (Shimadzu, Kyoto, Japan), by HPLC and then measured by the o-phtalaldehyde method [31].

4.11 Immunoprecipitation

PMN were starved for 30 min in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and then pulse-labeled with [^{35}S]methionine (10 μCi/ml) for the indicated periods. In the chase experiments, 1 mM cold methionine was added to the cells after pulse labeling and incubated for the indicated periods at 37°C. Immunoprecipitation with an anti-HDC antibody was performed as described previously [13]. The dried gel was analyzed using a Fujix BAS 2000 Bio-Imaging Analyzer.

4.12 Gelatin zymography

Gelatin zymography was performed according to the procedure described [32]. Briefly, SDS-PAGE was performed with 10% acrylamide slab gels containing 0.28% gelatin. After electrophoresis, gels were incubated twice in 2.5% Triton X-100 at room temperature for 30 min, and the in-gel gelatinase reaction was performed in 100 mM Tris-HCl pH 7.5 containing 10 mM $CaCl_2$ at 37°C for 12 h. Gelatin digestion was confirmed by Coomassie brilliant blue staining.

4.13 Streptolysin-O treatment

PMN were incubated with SLO (12,500 U/ml; preactivated by incubation for 15 min on ice with PBS containing 10 mM dithiothreitol) in PBS at 4°C for 10 min. After this binding step, cells were rinsed twice with PBS and then incubated at 37°C for 3 min to cause permeabilization. More than 80% of lactate dehydrogenase activity was recovered in the leaked fraction under these conditions. No immunoreactive band was detected in the leaked fraction upon immunoblot analyses with the anti-PDI antibody, indicating that the ER mem-

brane was intact. Detergent-free buffer was used in the immunofluorescence experiments for analysis of cells after selective permeabilization of the plasma membrane.

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Chemical Genetic Identification of the Histamine H1 Receptor as a Stimulator of Insulin-Induced Adipogenesis

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Summary

A large collection of bioactive compounds with diverse biological effects can be used as probes to elucidate new biological mechanisms that influence a particular cellular process. Here we analyze the effects of 880 well-known small-molecule bioactives or drugs on the insulin-induced adipogenesis of 3T3-L1 fibroblasts, a cell-culture model of fat cell differentiation. Our screen identified 86 compounds as modulators of the adipogenic differentiation of 3T3-L1 cells. Examination of their chemical and pharmacological information revealed that antihistamine drugs with distinct chemical scaffolds inhibit differentiation. Histamine H1 receptor is expressed in 3T3-L1 cells, and its knockdown by small interfering RNA impaired the insulin-induced adipogenic differentiation. Histamine receptors and histamine-like biogenic amines may play a role in inducing adipogenesis in response to insulin.

Introduction

The ongoing global explosion in the incidence of obesity has fueled efforts on molecular understanding of fat cell differentiation. The study of different steps leading to this terminal differentiation has been facilitated by the development of established preadipocyte cell lines. Among them, the 3T3-L1 fibroblast cell line is perhaps the best characterized, and its differentiation has served as an excellent cell-culture model of adipogenesis [1, 2]. When treated with insulin, 3T3-L1 cells undergo differentiation to mature fat cells, which are morphologically distinct from the original cells because of their rounded shapes and the presence of cytoplasmic oil droplets. The transparent morphology of 3T3-L1-derived adipocytes has been used for unveiling the molecular events that orchestrate adipogenesis, including the roles of C/EBPs and PPARγ in mediating the expression of adipocyte-specific genes [3, 4].

New biological pathways that influence particular cellular processes are often discovered by the use of bioactive small molecules. In the case of the adipogenic differentiation of 3T3-L1 cells, thiazolidinediones, dexamethasone, methylisobutylxanthine, HIV protease inhibitors, MAPK inhibitors, nonsteroidal anti-inflammatory drugs, and cyclosporin have been found to influence the differentiation [5–9]. These adipogenesis modulators facilitated studies of molecular cascades mediating adipogenesis, including the pathways of PPARγ, glucocorticoid receptor, CREB, MAPK, IKK, and NFAT. However, these agents were originally tested largely through empirical means, and more systematic analysis of small molecules could provide additional insights into the molecular events that influence adipogenic differentiation.

Here we analyze the effects of 880 bioactive small molecules on the insulin-induced adipogenesis of 3T3-L1 cells. The biological mechanisms or pharmacological effects of these molecules have been extensively studied, and many of them are marketed as pharmaceuticals. Just as DNA microarray analysis with annotated gene probes has provided insights into novel gene functions, the phenotypic adipogenesis assay with the annotated chemical library may permit quick elucidation of new biological mechanisms that influence adipogenic differentiation.

Results

Effects of 880 Compounds on Adipogenesis

We assayed a collection of 880 bioactive compounds for their ability to modulate the insulin-induced adipogenesis of 3T3-L1 cells. These commercially available bioactives (average molecular weight = 377) were selected for structural diversity and a broad spectrum covering therapeutic areas including neuropsychiatry, cardiology, immunology, anti-inflammatory, analgesia, cancer, metabolic diseases, etc. More than 85% of the compounds have been marketed either in the United States or Europe as pharmaceuticals or supplements, and their biological mechanisms or pharmacological effects have been extensively studied. The results of repeated phenotypic assays at 5 μM showed that 47 compounds stimulated adipogenesis while 39 compounds blocked differentiation without detectable cytotoxicity. Fifteen of 47 adipogenesis-enhancing chemicals were analogs of steroid hormones, consistent with the reported adipogenic effects of the glucocorticoid family of steroids [3]. The adipogenesis-enhancing compounds also included the Na^+ channel blockers, mexiletine and amiodarone, and the anticoagulants, acenocumarol and dicumarol. On the other hand, the 39 adipogenesis-blockers included antibiotics, agricultural chemicals, and nucleotide analogs. Figure 1 shows typical examples of morphological appearance and RT-PCR analysis of the chemical-treated cells. The cells treated with mifepristone (steroid), harmin (alkaloid), or homocystein thiolactone (amino acid analog) exhibited enhanced adipogenesis and increased expression of the adipogenic marker aP2. By contrast, the differentiation of cells incubated with isotretinoin (retinoic acid analog),

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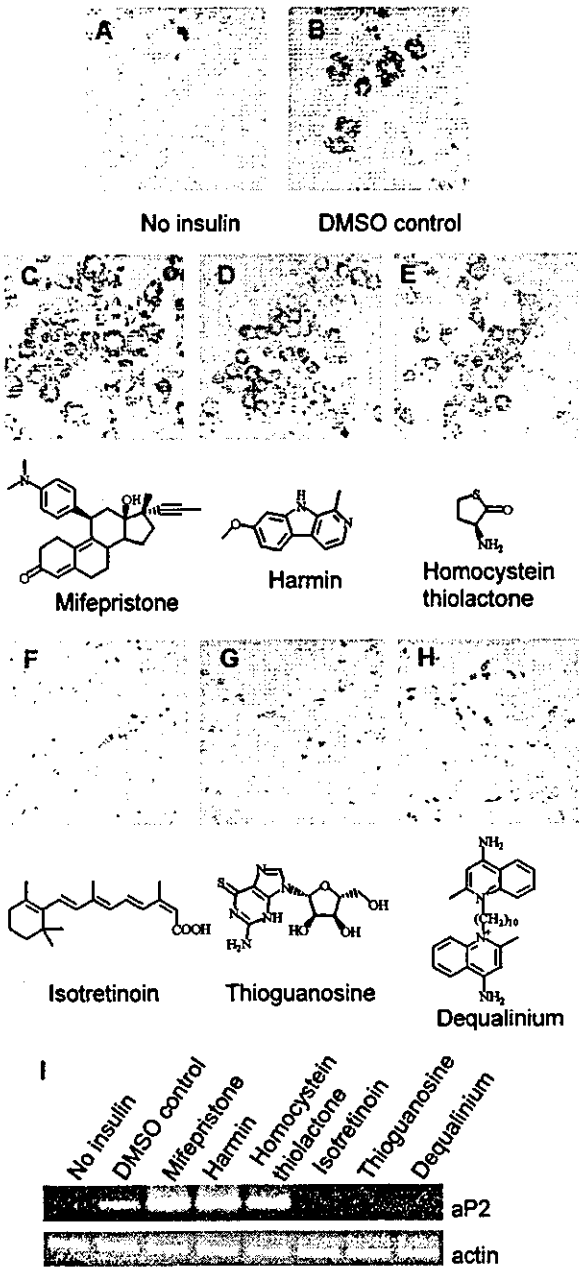

Figure 1. Effects of 880 Bioactive Compounds on Insulin-Induced Adipogenesis of 3T3-L1 Cells

The differentiation of 3T3-L1 cells was induced by insulin in the presence of 1% (v/v) DMSO only (B) or 5 μM compounds. Ten days after the induction, the differentiated cells were stained with Oil-Red O, and their microscopic images were captured. Effects of representative adipogenesis enhancers and blockers are shown: mifepristone (C), harmin (D), homocystein thiolactone (E), isotretinoin (F), thioguanosine (G), and dequalinium (H). (A) shows a microscopic image of the cells without insulin induction. The levels of differentiation were also evaluated by RT-PCR analysis of the aP2 gene, an adipocyte-specific marker (I).

thioguanosine (nucleotide analog), and dequalinium (antibiotic) was completely abolished, showing no oil droplets and no detectable increase of aP2 mRNA levels.

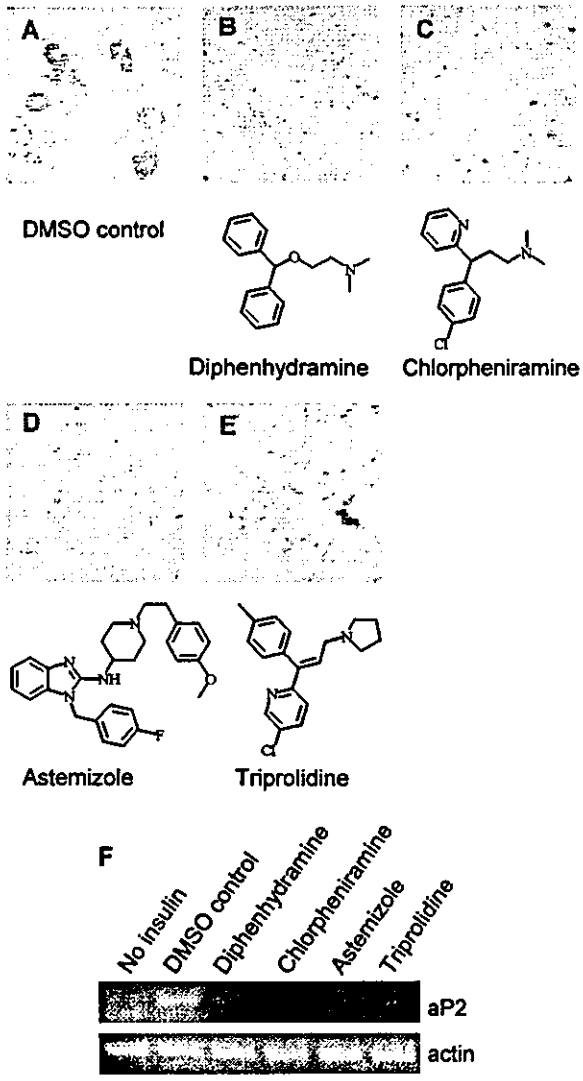

Figure 2. Inhibition of Adipogenic Differentiation by Histamine Blockers

The differentiation of 3T3-L1 cells was induced by insulin in the presence of 5 μM of histamine blockers: diphenhydramine (B), chlorpheniramine (C), astemizole (D), and triprolidine (E). Ten days after the induction, the differentiated cells were stained with Oil-Red O, and their microscopic images were captured. (A) shows a microscopic image of the cells treated with 1% (v/v) DMSO only. The levels of differentiation were also evaluated by RT-PCR analysis of the aP2 gene, an adipocyte-specific marker (F).

While performing the adipogenesis assays, we noted persistent adipogenesis-blocking effects of histamine H1 receptor antagonists. Incubation with any one of the four clinically used histamine H1 receptor blockers, diphenhydramine, chlorpheniramine, astemizole, and triprolidine completely abolished the differentiation of 3T3-L1 cells, resulting in no accumulation of triacylglycerol vesicles and impaired expression of the aP2 marker (Figure 2). Chemically analogous monoamine drugs with no antihistamine activity such as dacarbazine had little effect on adipogenic differentiation (data not shown), suggesting that the inhibition of adipogenesis is due to histamine antagonism.

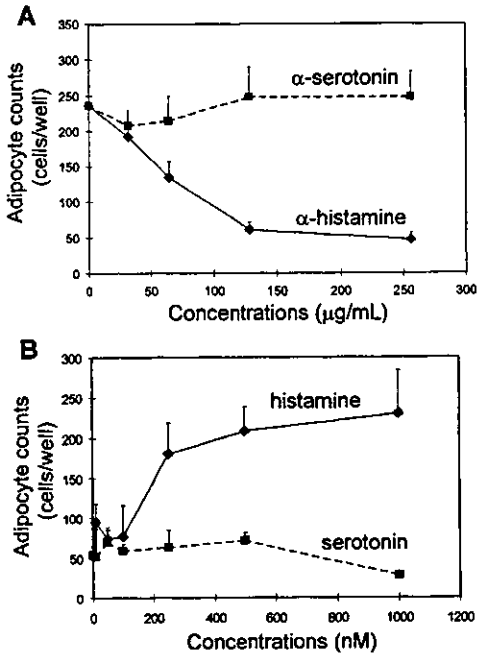

Figure 3. Inhibition of Adipogenic Differentiation by an Anti-Histamine Antibody

The differentiation of 3T3-L1 cells was induced by insulin in the presence of anti-histamine or anti-serotonin antibodies. Ten days after the induction, the differentiated cells were stained with Oil-Red O and counted (A). Addition of free histamine to the medium in the presence of anti-histamine antibody restored the insulin-induced adipogenesis, while that of serotonin had no detectable effect (B). Mean values and SE of three independent experiments were plotted.

Histamine as an Adipogenic Factor for 3T3-L1 Cells

Histamine, released from mast cells, basophils, or enterochromaffin-like cells, circulates in the blood, with normal mouse plasma containing a high nM range of histamine [10]. We quantified the histamine concentration in the fetal bovine serum that we used for the differentiation of 3T3-L1 cells and found that it contained 14.1 nM histamine, which is higher than the estimated K_d of histamine for the histamine H1 receptor [11]. We speculated that histamine in the fetal bovine serum acts as a stimulatory factor for the adipogenic differentiation of 3T3-L1 cells. To test this hypothesis, we used anti-histamine antibody to neutralize histamine in the culture medium. 3T3-L1 cells were treated with insulin in the presence of anti-histamine antibody, and the numbers of differentiated cells were counted after staining with Oil-Red O. The neutralization of serum histamine resulted in a decreased number of differentiated cells in a dose-dependent manner. In contrast, an anti-serotonin antibody had no detectable effect on adipogenic differentiation even at high concentration (Figure 3A). Addition of free histamine back into the medium restored the level of adipogenesis, whereas adding serotonin had no effect (Figure 3B). These results suggest that histamine in the serum potentiates the insulin-induced differentiation.

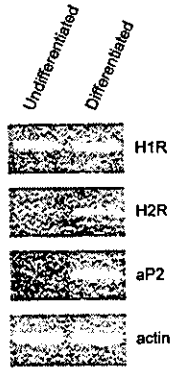

Figure 4. Expression Levels of the Histamine H1 and H2 Receptors in Undifferentiated and Fully Differentiated 3T3-L1 Cells

Total RNA was extracted from undifferentiated 3T3-L1 fibroblasts or fully differentiated counterparts, and subjected to RT-PCR analysis with primers specific for the histamine H1 receptor, the histamine H2 receptor, aP2, and β-actin. The histamine H1 receptor is expressed both in undifferentiated and differentiated 3T3-L1 cells, while expression of histamine H2 receptor was detected only after differentiation.

Expression of the Histamine H1 Receptor in 3T3-L1 Cells

We next examined the expression levels of histamine receptors in 3T3-L1 cells. Four members of the histamine receptor family have been described so far (H1, H2, H3, and H4 receptors) [12, 13]. RT-PCR analysis of each receptor gene demonstrated that the histamine H1 receptor is highly expressed both in 3T3-L1 cells and their fully differentiated counterparts (Figure 4). Expression of the H2 receptor was detected only after differentiation (Figure 4), and expression of the H3 and H4 receptors was observed neither before nor after differentiation (data not shown). Diphenhydramine, chlorpheniramine, astemizole, and triprolidine are well-known blockers selective for histamine H1 receptor [12]: the expression of the H1 receptor in 3T3-L1 cells is consistent with the observed inhibitory effects of H1-receptor-selective antagonists.

Knockdown of the Histamine H1 Receptor Impairs the Adipogenesis of 3T3-L1 Cells

It is possible to imagine that H1 antagonists inhibit adipogenesis by targeting proteins other than the histamine H1 receptor. For example, diphenhydramine, chlorpheniramine, and triprolidine have been reported to inhibit cytochrome P-450 2D family members, as well as the histamine H1 receptor (14). To gain direct evidence that the histamine H1 receptor plays a role in inducing the adipogenic differentiation of 3T3-L1 cells, we employed the small interfering RNA (siRNA) technique [15]. An oligonucleotide duplex encoding an siRNA specific for the histamine H1 receptor was inserted into an expression vector driven by the RNA polymerase III H1 gene promoter [16], and the resulting vector was stably transfected into 3T3-L1 cells. Neomycin-resistance selection established two stably transfected clones. RT-PCR analysis of the clones confirmed selective silencing of

histamine H1 receptor expression (Figure 5A). The knockdown of the histamine H1 receptor rendered the two clones resistant to insulin-induced adipogenesis (Figures 5B–5H): no oil droplets or expression of the aP2 gene was observed after insulin treatment, while 20%–30% cells of 3T3-L1 neo, a transformant with the empty vector, differentiated just as well as the parental 3T3-L1 cells. These results indicate that the histamine H1 receptor is required for the facile, efficient adipogenesis of 3T3-L1 cells.

Discussion

Histamine and Insulin

Histamine is a chemical mediator implicated in inflammation, gastric acid secretion, and neurotransmission [17–21] and its antagonists are excellent pharmaceuticals for allergy and gastric ulcer [22, 23]. Our study identified histamine blockers as inhibitors of the insulin-induced differentiation of 3T3-L1 cells and suggested a role for the histamine H1 receptor in promotion of insulin-induced adipogenesis. Roles of histamine in food intake and adiposity have been demonstrated using a range of animal models. In whole animals, however, disruption of the histamine H1 receptor generates obese phenotypes instead of decreased levels of body fat [24]. The obese phenotypes are believed to result primarily from disabled neuronal function of histamine and thereby inhibition of leptin, a circulating satiety factor that suppresses food intake [24, 25]. Phenotypes of knockout animals are often governed by systemic, global effects of gene function in the context of complex interplay of related proteins: the adipogenic effects of the histamine H1 receptor, which were detectable in cultured cells, may be masked and undetectable in the knockout mice. It is also possible that the adipogenic effects of the histamine H1 receptor or histamine are exerted transiently or under particular conditions in vivo and might be pronounced only in the in vitro model of adipogenesis.

Our results can also be explained by considering histamine as a general stimulatory factor of insulin, as the inhibition of histamine rendered 3T3-L1 cells completely nonresponsive to insulin for adipogenesis. The stimulatory role of histamine is consistent with the glucose intolerance and insulin resistance reported recently in knockout mice of histidine decarboxylase, the rate-limiting enzyme for histamine synthesis in mammals [26]. The blood glucose levels in these mice are not responsive to injected insulin, and symptoms of hyperinsulinemia have been observed. Moreover, it has recently been reported that histamine stimulates glucose uptake in rat adipocyte [27] and that insulin up-regulates expression of the histamine H1 receptor in human astrocytoma cells [28]. These previous observations and ours all suggest that histamine and its receptors play a role in controlling insulin function and its resistance. However, it remains unclear how histamine modulates insulin function. The insulin-induced differentiation of 3T3-L1 cells may find use as an in vitro phenotypic model for understanding the potential role of histamine and its receptors in further detail.

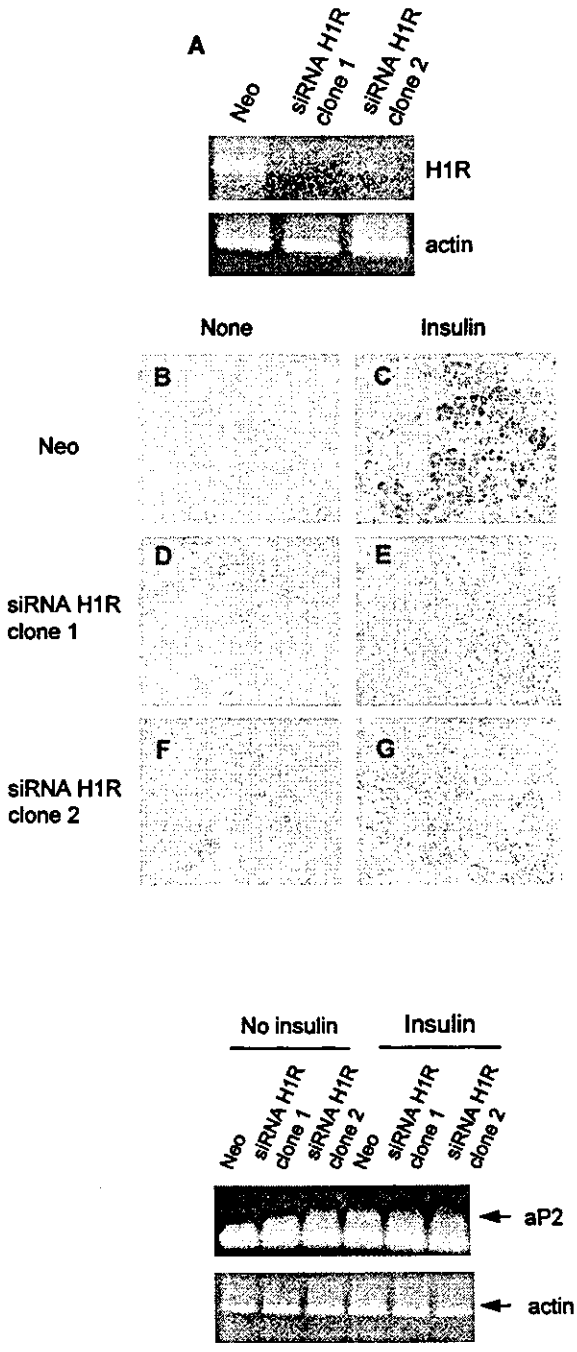

Figure 5. Inhibition of Insulin-Induced Adipogenesis by siRNA Knockdown of the Histamine H1 Receptor

3T3-L1 cells were transfected with an expression vector encoding an siRNA specific for the histamine H1 receptor gene, and two stably transfected clones were obtained. RT-PCR analysis of the clones demonstrated the successful knockdown of the histamine H1 receptor in both clones (A). When induced by insulin, approximately 20% of 3T3-L1 neo cells exhibited phenotypes of adipocytes (B and C), whereas the knockdown clones were resistant to insulin-induced differentiation (D–G). The levels of differentiation were also evaluated by RT-PCR analysis of the aP2 gene, an adipocyte-specific marker (H).

Adipogenesis for Monitoring Histamine

In our previous work, we profiled a chemical library of 10,000 divergent synthetic compounds, using the adipogenic differentiation of 3T3-L1 cells. Differentiation profiling enabled the construction of a smaller focused library of bioactive molecules, from which we were able to isolate small molecules with a range of pharmacological effects [29]. The results suggested that insulin-induced adipogenesis serves as a phenotypic indicator of seemingly unrelated pharmacological effects of chemicals. Here we profiled 880 "known" drugs by adipogenesis in search of therapeutically important signaling pathways that modulate insulin-induced adipogenesis. The screening results, followed by siRNA validation, show that histamine blockers impair the differentiation of 3T3-L1 cells through their inhibition of the histamine H1 receptor. The insulin-induced adipogenesis of 3T3-L1 cells may prove to be a convenient phenotypic assay for the analysis of the histamine H1 receptor pathway or its chemical modulators.

Use of an Annotated Chemical Library to Elucidate Biological Functions

Bioactive small molecules, whether natural products or synthetic, have served as a powerful probe for the study of protein function in cells. Among prominent examples are the immunosuppressive drug FK506, the microtubule poison colchicine, the tumor promoter phorbol esters, the histone-deacetylase inhibitor trichostatin A, the kinase inhibitor wortmannin, and the proteasome inhibitor lactacystin [30–33]. These probes have been extensively used for the biological studies by virtue of their convenient handling, high cell permeability, and conditional nature of their chemical effects. A large collection of such biologically proven compounds would constitute a library of annotated chemical probes that could permit quick elucidation of new biological mechanisms [34].

Our case study supports the proposed utility of large libraries of annotated chemical probes for biological studies. However, the chemical library used in this study is still insufficient in its number and diversity: it consists primarily of off-patent drugs and widely known bioactives. Fortunately, recent advances in chemical diversity generation and new screening methodologies are increasing the number of unique bioactive compounds with novel mechanisms [35]. Once the newly discovered agents have been more completely studied through further selectivity validation or target identification, they will supplement the array of biologically active molecules that are currently available and might constitute a chemical library that can probe a more complete set of gene products and pathways. Target identification of bioactive compounds, whether synthetic or natural products, will increasingly be important for future endeavors because newly discovered agents can never be useful for the type of applications described here until they have been annotated.

The greatest challenge of the chemical approach remains the issue of specificity. A number of excellent small-molecule drugs, especially synthetic ones, target multiple cellular proteins to achieve synergistic pharmacological effects and to allow application in broader conditions. One needs to ensure that the phenotype caused by a compound is indeed due solely to the inhibition of its supposed target. Confirmation using independent approaches is required after an initial chemical screening, and the siRNA technique may be an excellent companion when a phenotype-causing probe inhibits a protein's function. In our study, a correlation between histamine-blocking activity and adipogenesis inhibition was found in multiple compounds and served as a basis for the successful siRNA analysis of the histamine H1 receptor. Our results suggest that careful examination of the structure-activity relationship of phenotype-causing probes relieves the specificity concern and leads to a tractable number of hypotheses for more time-consuming siRNA confirmation.

Significance

Systematic elucidation of protein function by small-molecule probes is referred to as reverse chemical genetics [36]. This interdisciplinary approach uniting biology, chemistry, and pharmacology still has problems to overcome for its full potential to be reached, but enriches future opportunities in biology and medicine. Our case study, although at an early stage of the field, provides an encouraging example for the forthcoming endeavor and suggests an interesting role for histamine receptors in insulin-induced adipogenesis.

Experimental Procedures

Materials

3T3-L1 cells were obtained from ATCC and maintained in DMEM supplemented with 10% calf serum. Anti-histamine or serotonin antibodies were from CHEMICON and ImmunoStar, respectively. A collection of 880 bioactive compounds (Prestwick Chemical Library) was purchased from Prestwick Chemical. Twenty-five milligrams of dry powder of each compound were dissolved in DMSO and stored in dark at −20 C° before use.

Chemical Screen of the Adipogenic Differentiation

3T3-L1 cells were grown to complete confluence and incubated for another two days. The medium was switched to DMEM containing 10% fetal bovine serum, 5 μg/ml of insulin, and 5 μM of each bioactive compound. The final DMSO concentration was 1% (v/v). Three days after the induction of adipogenic differentiation, half of the medium was changed to fresh medium without chemicals every two days. Adipose oil droplets were stained with Oil-Red O ten days after the chemical treatment, and the cells were examined under microscope. This procedure permits the adipogenesis of 15%–20% of the cells in the absence of a chemical (DMSO control), enabling the discovery of both adipogenesis enhancers and blockers.

RT-PCR

Total RNA extraction and reverse transcription reaction were performed as described [28]. Primer sets used for PCR were as follows. The histamine H1 receptor: 5′-CTG GTG GTG GTT CTT AGT AGT ATC-3′ (sense) and 5′-CAG CAT CAG CAA AGT GGG GAG GTA-3′ (antisense). The histamine H2 receptor: 5′-CGT CTG CCT GGC TGT CAG CTT G-3′ (sense) and 5′-AGA GGC AGG TAG AAG GTG ACC A-3′ (antisense). The histamine H3 receptor: 5′-CTC TGC AAG CTG TGG CTG GTG GTA GAC TAC CTA CTG TGT G-3′ (sense) and 5′-CTT CTT GTC CCG CGA CAG CCG AAA GCG CTG GGT GAT GCT T-3′ (antisense). The histamine H4 receptor: 5′-CAC GCT GTT TAA CTG GAA TTT TGG AAG TGG AAT CTG CAT G-3′ (sense) and 5′-ACC AAG AAA GCC AGT ATC CAA ACA GCC ACC ATT TGA GC-3′ (antisense). Beta-actin: 5′-CGT ACC ACC GGC ATT GTG AT-3′

(sense) and 5′-GAG CAG TAA TCT CCT TCT GC-3′ (antisense). The primers for the aP2 gene were described in our previous study [29]. PCR samples were denatured at 94°C for 40 s, annealed at 60°C for 40 s, and extended at 72°C for 60 s with 22 cycles for β-actin, 24 cycles for aP2, and 28 cycles for the histamine H1 and H2 receptors.

Quantification of Histamine

Fetal bovine serum (Invitrogen) was treated with 3% $HClO_4$ and centrifuged at 800 × g for 5 min. The supernatant was added to an equal volume of 0.5 M sodium phosphate buffer (pH 6.5) and the pH of the mixture was adjusted to 6.5 by adding 5 N KOH. Histamine in the sample was separated by an Amberlite CG-50 cation-exchange column [37] and quantified by a fluorometric assay as described [38].

siRNA Experiment

To target the mRNA of the histamine H1 receptor, we designed two complimentary oligonucleotides, 5′-GAT CCC CGA TCA TGA CCG CCA TCA TCT TCA AGA GAG ATG ATG GCG GTC ATG ATC TTT TT-3′ and 5′-AGC TAA AAA GAT CAT GAC CGC CAT CAT CTC TCT TGA AGA TGA TGG CGG TCA TGA TCG GG-3′. The underlined sequences indicate a target sequence (position 574–593) and its reverse complement. The oligonucleotides were annealed and then inserted into a pSUPER.neo vector (Oligoengine). The resulting plasmid was transfected into 3T3-L1 cells with Lipofectamine Reagent (Invitrogen). To establish stably transfected clones, neomycin-derivative G418 (Gibco) was used at a concentration of 500 μg/ml and two stable transformants were established. The expression levels of the histamine H1 receptor were evaluated by RT-PCR.

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