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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1150

An emergency operation was performed which revealed foreign material which had penetrated into the ileum. A wedge resection of the perforated bowel region was undertaken, and intraperitoneal drainage was performed. The patient was discharged from our hospital nine days post-operatively in good condition.

The object that had been imaged on the computed tomography scan was found to be the foot of a soft shelled turtle (fig 2), commonly referred to as "Supon" in Japanese (scientific name *Trionyx sinensis*). This turtle is only served on special occasions and is an expensive item for cuisine. Discussions with the patient indicated that he had eaten soft shelled turtle two months before the operation during a new year festival in January. As an aid in identifying this type of situation, it is important to also make use of preoperative computed tomography scans, review the patient's history in light of any prior operations and, where possible, evaluate the patient's menu or discuss with the family to recollect any sources of hard body parts that could be an immediate source of the problem.

Figure 2 A picture of the foot of a soft shelled turtle.

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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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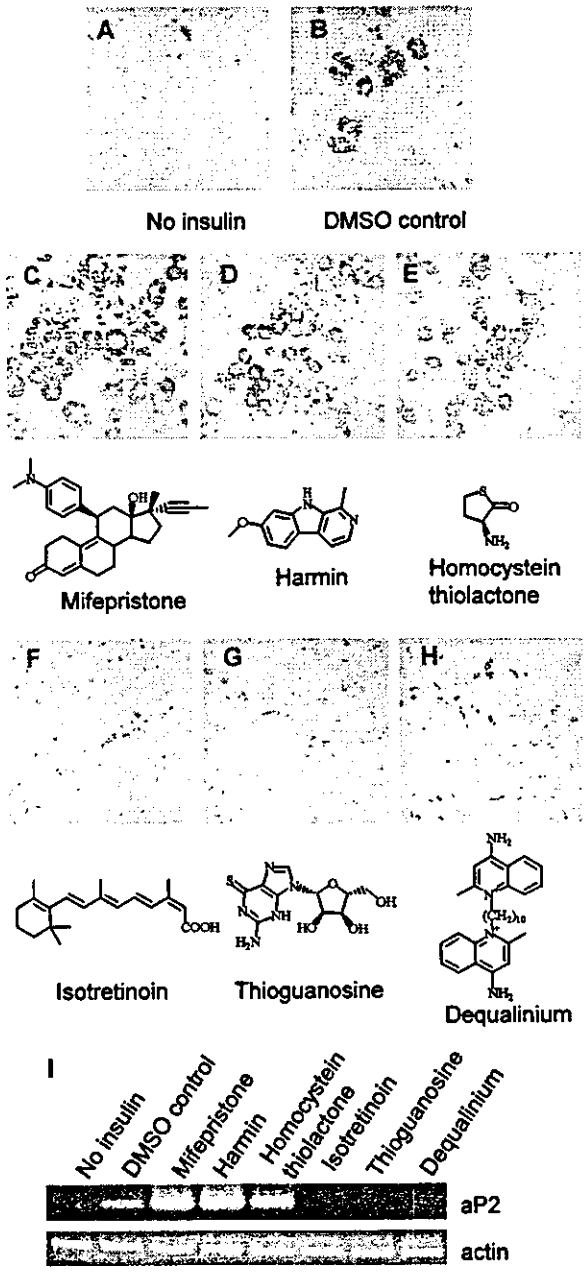

Figure 1. Effects of 880 Bioactive Compounds on Insulin-Induced Adipogenesis of 3T3-L1 Cells

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.

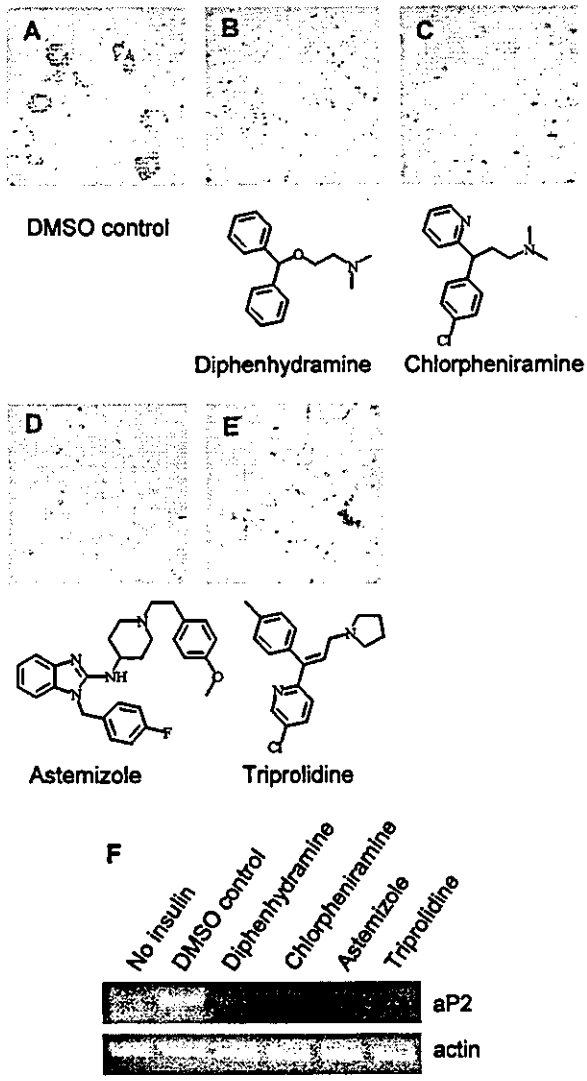

Figure 2. Inhibition of Adipogenic Differentiation by Histamine Blockers

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.

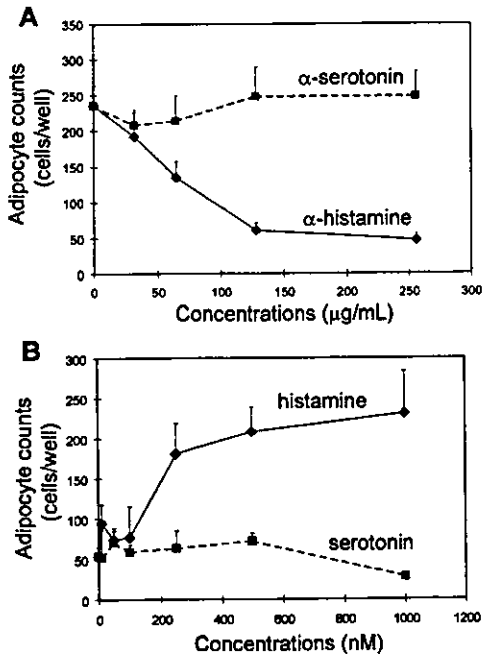

Figure 3. Inhibition of Adipogenic Differentiation by an Anti-Histamine Antibody

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.

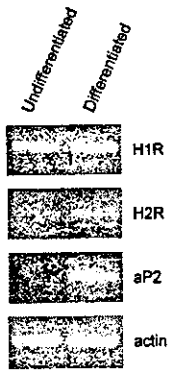

Figure 4. Expression Levels of the Histamine H1 and H2 Receptors in Undifferentiated and Fully Differentiated 3T3-L1 Cells

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.

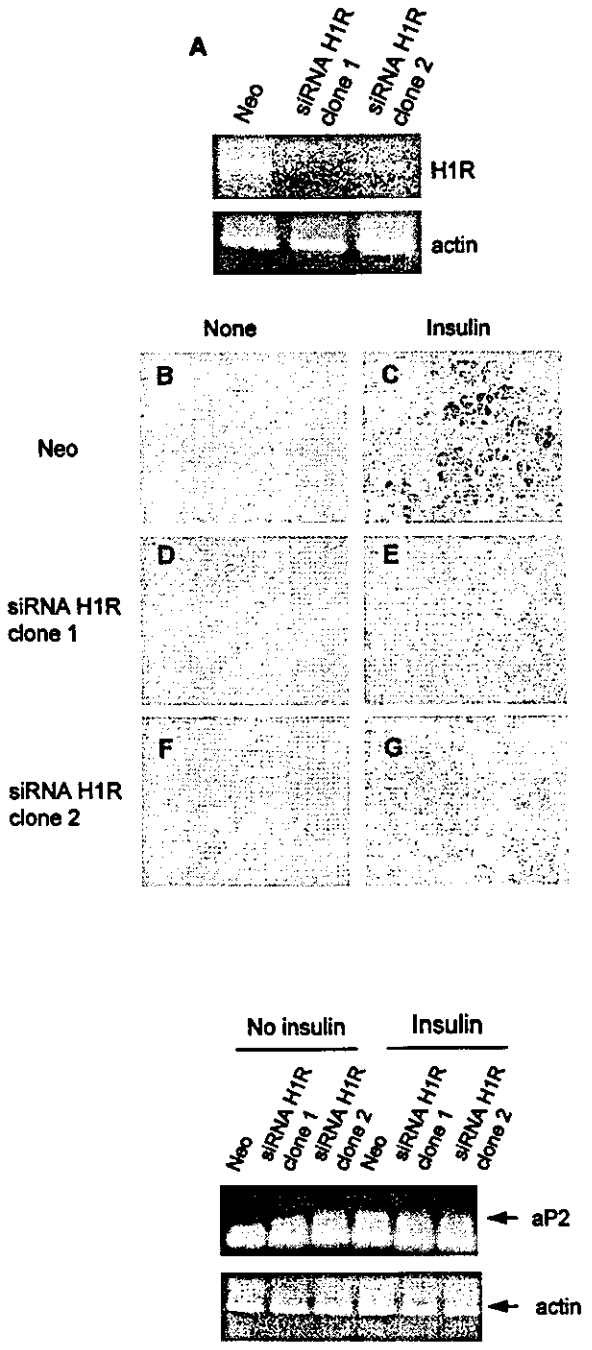

Figure 5. Inhibition of Insulin-Induced Adipogenesis by siRNA Knockdown of the Histamine H1 Receptor

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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Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation

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Abstract

Prostaglandins (PGs) have been shown to play various roles in adipogenesis. In this study, we investigated on which PGE receptor subtypes are involved in the inhibition of 3T3-L1 preadipocyte differentiation. The triglyceride content of cells, used as an index of differentiation, was decreased when PGE_2, the FP-agonist fluprostenol or dibutyryl cAMP, was exogenously added to differentiation cocktails. 3T3-L1 preadipocyte cells express mRNAs for the prostanoid EP4, FP, and IP receptors. PGE_2 and the EP4 agonist AE1-329 increased cAMP levels in preadipocytes in a dose-dependent manner. AE1-329 suppressed the expression induction of differentiation marker genes such as resistin and peroxisome proliferator-activated receptor-γ. The inhibitory effect of PGE_2 but not that of fluprostenol was reversed by the addition of the EP4 antagonist AE3-208. AE3-208 mimicked the differentiation-promoting effects of indomethacin. These results suggest that the EP4 receptor mediates the suppressive action of PGE_2 in 3T3-L1 adipocyte differentiation.

Keywords: Prostanoid; Receptor subtypes; Adipogenesis; Fat cell; Aspirin-like drugs

Adipogenesis is a crucial aspect in controlling body fat mass [1,2]. Acquisition of the mature adipocyte phenotype is a highly regulated process in which preadipocytes undergo differentiation, resulting in both an increase in size and number of mature adipocytes in adipose tissue. It has been shown that cyclooxygenase (COX) products such as prostaglandin (PG) E_2 and $PGF_{2\alpha}$ inhibit adipocyte development [3,4]. A recent study suggested that COX-2 might be involved in body fat regulation [5]. Mice heterozygous for the COX-2 gene showed approximately 30% increased body weight, with 2 to 3-fold larger fat pads compared with those of wild-type animals. PGE_2 production in adipose tissue from COX-2 null mice was only 20% of that of wild-type mice. These results suggested that COX-2 as well as PGE_2 participates in the negative regulation of adipocyte differentiation.

PGs exert a wide range of actions through their binding to plasma membrane receptors [6,7]. $PGF_{2\alpha}$ exerts its actions via a specific interaction with the type F prostanoid receptor FP which activates phospholipase C, resulting in phosphatidylinositol breakdown [8]. In contrast, PGE_2 exerts its actions through its interaction with four PGE_2 receptor subtypes (EP; EP1, EP2, EP3, and EP4). The EP subtypes differ in their signal transduction pathways; EP1 is coupled to the mobilization of intracellular $[Ca^{2+}]$, EP2, and EP4 are coupled to the stimulation of adenylyl cyclase, and EP3 is mainly coupled to inhibition of adenylyl cyclase. The diverse actions of PGE_2 can be explained by the existence of these multiple EP subtypes with different signal transduction pathways [9,10]. Due to the lack of subtype-specific agonists and antagonists, the involvement of each EP

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subtype in a specific PGE_2 action including suppression of adipocyte differentiation has not been well established. The current study was undertaken to determine which EP subtype participates in the negative regulation of adipocyte differentiation. 3T3-L1 cells were used as a model system and a pharmacological approach using a highly selective EP agonist and antagonist was employed to determine the relative contributions of the EP receptor subtypes.

Materials and methods

Reagents. Dibutyryl cAMP, dexamethasone, and indomethacin were purchased from Sigma (St. Louis, MO). The ^{125}I-labeled cyclic AMP assay system was purchased from Amersham Biotech (Piscataway, NJ). PGE_2 was purchased from Funakoshi (Tokyo, Japan), and fluprostenol was from Cayman Chemical (Ann Arbor, MI). DI-004 (an EP1 agonist), AE1–259 (an EP2 agonist), AE1–329 (an EP4 agonist), and AE3–208 (an EP4 antagonist) were generous gifts from ONO Pharmaceuticals (Osaka, Japan) [11–13]. Oligonucleotides were from Invitrogen (Carlsbad, CA). All other chemicals were commercial products of reagent grade.

Cell culture, differentiation, and measurement of triglyceride content. 3T3-L1 preadipocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine. Differentiation was initiated by culturing the cells in differentiation medium which contained 10% FBS, 4 mM glutamine, 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone, and 5 μg/ml insulin. After two days, the culture medium was changed to adipocyte growth medium containing 10% FBS, 4 mM glutamine, and 5 μg/ml insulin and exchanged every two days for an additional six days. Reagents such as PG agonists and the PG antagonist were added to both the differentiation medium and adipocyte growth medium as required. For measurements of triglyceride content, cells grown in six-well plates were harvested in 1 ml of 2-propanol, sonicated, and triglyceride levels in the cell lysate were measured using Triglyceride G Test Kit (Wako, Tokyo, Japan). For Oil Red O staining, cells were washed in PBS, fixed in 3.7% formaldehyde for 10 min, and followed by staining with Oil Red O for 1 h. Oil Red O was prepared by diluting a stock solution (0.5 g of Oil Red O (Sigma) in 100 ml of 2-propanol) with water (6:4) followed by filtration.

cAMP formation assay. cAMP levels in 3T3-L1 cells on day 0 and day 2 of the differentiation program were determined as reported previously [14]. Cells cultured in 24-well plates (1×10^6 cells/well) were washed twice with 0.5 ml Krebs–Hepes buffer (pH 7.4) with 10 μM indomethacin, and preincubated in this solution for 10 min. Reactions were started by the addition of test reagents along with 100 μM Ro-10-1724 and 10 μM indomethacin. After incubation for 10 min at 37 °C, reactions were terminated by the addition of 10% trichloroacetic acid. The cAMP content of the cells was then measured using a cAMP radioimmunoassay kit.

[^{3}H]PGE_2 binding assay. 3T3-L1 preadipocytes grown to confluency were homogenized with a Potter-Elvehjem homogenizer in 10 mM Mes/NaOH, pH 6.0, containing 10 mM $MgCl_2$, 1 mM EDTA, 20 μM indomethacin, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 250,000g for 10 min, the pellet was washed and suspended in the same buffer. The membrane fraction (200 μg) was incubated with 4 nM [^{3}H]PGE_2 at 30 °C for 1 h, and [^{3}H]PGE_2 bound to the membrane fraction was determined by adding a 1000-fold excess of unlabeled PGE_2 to the incubation mixture. Specific binding was calculated by subtracting the non-specific binding from the total binding.

RNA isolation and semi-quantitative RT-PCR. Total cellular RNA was isolated from 2×10^6 3T3-L1 cells on the indicated day of the differentiation program by the acid guanidinium thiocyanate–phenol–chloroform method [15]. Cells collected just before being cultured in fresh differentiation cocktail were collected as the sample at time 0 h. To examine the differentiation-dependent gene expression of peroxisome proliferator-activated receptor-γ (PPARγ) and resistin, semi-quantitative reverse transcription PCR (RT-PCR) was performed. Complementary DNA was synthesized from total RNA (10 μg) using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using a GeneAmp 9700 (Perkin–Elmer Applied Biosystems, Foster City, CA). Primers and PCR conditions used in the PCR for EP1, EP2, EP3, EP4, IP, and FP genes have been described previously [16]. Primers used for PPARγ, resistin, and β-actin were as follows: PPARγ, 5′-ttcccagcatttctgctccacactatgaag-3′ (sense), 5′-cggcagttaagatcacacctatcataaata-3′ (antisense); resistin, 5′-aatgcaataaagaacattggc-3′ (sense), 5′-aggtgcctgtagagaccggag-3′ (antisense); and β-actin, 5′-accaactgggacgacatggagaagatctgg-3′ (sense), 5′-ccggccagccaggtccagacgcaggatggc-3′ (antisense). All PCRs were confirmed to be in the logarithmic phase by monitoring the products obtained at the indicated number ±2 cycles. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR experiments were independently repeated three times.

Results

Effects of fluprostenol, PGE_2, and dibutyryl cAMP on adipocyte differentiation

3T3-L1 preadipocyte cells were primed with insulin, dexamethasone, and IBMX for two days followed by treatment with insulin for an additional six days. Their differentiation into adipocytes was monitored by Oil Red O staining, and furthermore their triglyceride (TG) content was measured as an index of differentiation (Figs. 1A and B). Indeed, the adipocytes contained 80.4 ± 2.94 mg TG/plate (2.0×10^6 cells/plate), but the 3T3-L1 cells cultured in the absence of the differentiation cocktail exhibited only 3.45 ± 0.15 mg TG/plate (data not shown). As previously reported, when the differentiation program was performed in the presence of 0.1 μM of fluprostenol, an FP-agonist, the positive area stained with Oil Red O was greatly reduced and the TG content was reduced to approximately one-fifth of the control level (Figs. 1A and B). Similarly, PGE_2 (0.1 μM) as well as the membrane-permeable cAMP, dibutyryl cAMP, markedly reduced the Oil Red O-stained regions and TG content in 3T3-L1 cells (Figs. 1A and B).

Expression of prostanoid EP4 receptors during adipocyte differentiation

We next examined the mRNA expression of prostanoid receptors in 3T3-L1 cells during the differentiation program (Fig. 2). As previously reported [17], the FP and IP mRNAs were expressed during the differentiation period. In addition, among the PGE receptor subtypes, significant expression of EP4 mRNA was de-

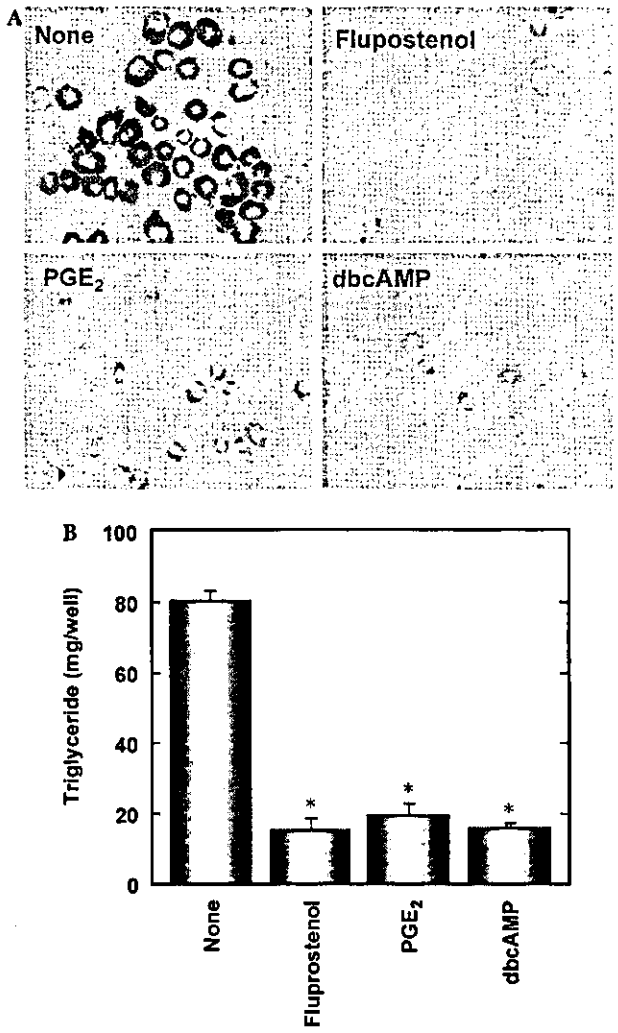

Fig. 1. PGE_2 and dibutyryl cAMP suppress 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with the FP-agonist fluprostenol (0.1 μM), PGE_2 (0.1 μM) or dibutyryl cAMP (dbcAMP) (1 mM). On day 8, the cells were subjected to Oil Red O staining (A), or suspended in 2-propanol, and their triglyceride content was determined as described in Materials and methods (B). Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus vehicle (Student's t test).

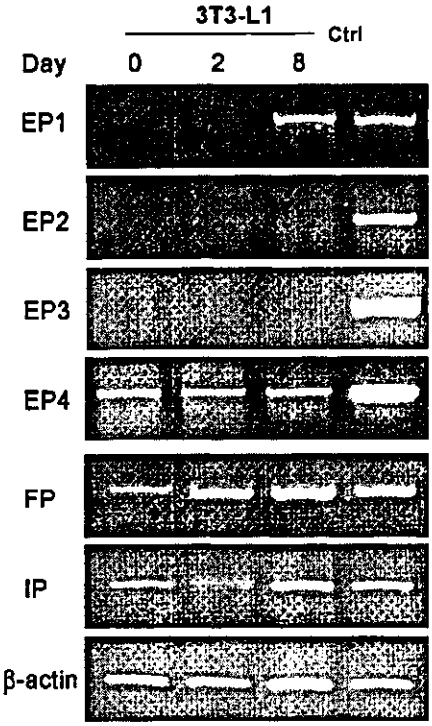

Fig. 2. RNA expression of prostanoid receptors during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were treated with a standard differentiation cocktail as described in Materials and methods. Total RNA was extracted from untreated cells (day 0), cells on day 2 (day 2) or cells on day 8 (day 8) of the differentiation program. Total RNA was subjected to RT-PCR analysis. Mouse lung RNA was used as a positive control for EP2, EP4, IP, and β-actin, and mouse kidney RNA was used for EP1, EP3, and FP (Ctrl). The experiments were independently repeated three times and similar results were obtained.

tected throughout the differentiation process. We failed to detect a significant amount of EP2 and EP3 receptor mRNA in these cells. Expression of EP1 mRNA was undetectable in the untreated cells and in the cells on day 2, but could be detected in cells on day 8. When we performed the $[^3H]PGE_2$ binding assay with a crude membrane fraction of undifferentiated 3T3-L1 preadipocytes, we detected a specific binding with a value of 15.1 ± 1.6 fmol/mg protein, and this binding was inhibited by more than 70% in the presence of an excess of the EP4-specific agonist AE1–329, but not in the presence of an excess of the EP2-specific agonist AE1–259. Based on these results, EP4 appears to be the EP receptor predominantly expressed in 3T3-L1 preadipocytes. We then performed the cAMP formation assay in 3T3-L1 preadipocytes (Fig. 3). PGE_2, but not fluprostenol, significantly increased cAMP formation in these cells in a dose dependent manner. This effect of PGE_2 was mimicked by AE1–329, but not by AE1–259. These results indicate that 3T3-L1 preadipocytes express functional EP4 receptors coupled to the stimulation of cAMP production.

Effects of an EP4 agonist on gene expression of resistin and PPARγ during adipocyte differentiation

When we investigated the effects of EP-selective agonists on adipocyte differentiation, AE1–329 mimicked the inhibitory effect of PGE_2, but DI-004, an EP1 agonist, failed. These results indicated that PGE_2 inhibits adipocyte differentiation by acting on EP4 receptor (Fig. 4A). We then examined the effects of an EP4 agonist on differentiation-dependent gene expression of resistin and PPARγ in 3T3-L1 cells (Fig. 4B). The mRNA for resi-

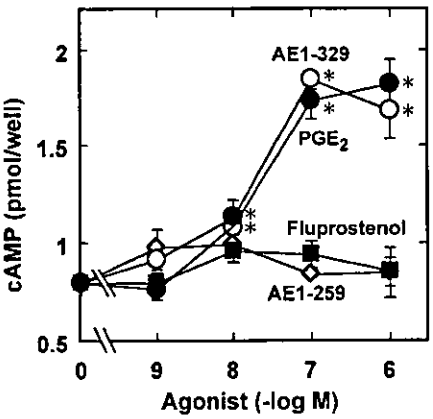

Fig. 3. Effects of PG agonists on cAMP accumulation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes (1×10^6 cells/well) were stimulated with the indicated concentrations of PGE_2, an EP4 agonist (AE1-329), an EP2 agonist (AE1-259), and fluprostenol for 10 min at 37 °C. cAMP formed was measured by radioimmunoassay. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus the basal cAMP level (Student's t test).

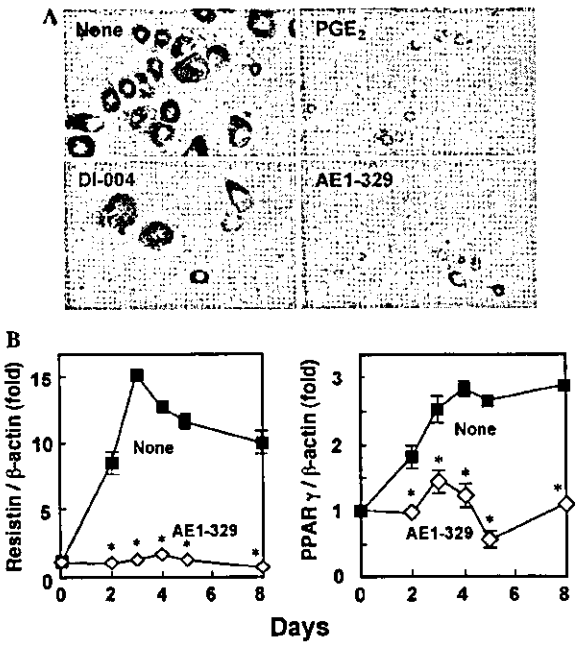

Fig. 4. Effects of an EP4 agonist on adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with PGE_2, DI-004 or AE1-329 (0.1 μM for each reagent). On day 8, the cells were subjected to Oil Red O staining (A). Alternatively, 3T3-L1 cells were stimulated with a standard differentiation cocktail in the presence or absence of AE1-329 (0.1 μM). Total RNA was isolated on the indicated day of the differentiation program, and subjected to semi-quantitative RT-PCR analysis as described in Materials and methods (B). The resistin and PPARγ mRNA levels were normalized to the β-actin mRNA levels and the data are represented as the fold of the value on day 0. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

stin, a hormone linking obesity to diabetes in rodents [18], was only weakly expressed in preadipocytes, but was drastically induced during the first three days of differentiation and its expression remained greater than 10-fold of the basal level until day 8. AE1–329 (0.1 μM) completely inhibited this increase in expression levels, resulting in an almost unchanged level of expression throughout the differentiation period. The mRNA for PPARγ, a transcription factor playing a central role in differentiation [19–21], was induced by 2-fold on the second day, and was subsequently maintained at high levels of greater than 2.5-fold of the basal expression. AE1–329 completely inhibited the increase observed on day 2, and suppressed the expression of PPARγ mRNA to within 1.5-fold of the basal level throughout the differentiation period. Such inhibition of resistin and PPARγ gene expression by AE1–329 was reproduced by PGE_2, but not by AE1–259 (data not shown). These results suggest that EP4 is the receptor responsible for the suppressive effects of PGE_2 on adipogenesis.

PGE_2-elicted but not FP-agonist-elicited inhibition of differentiation is mediated via EP4 receptor activation

It has been reported that FP receptor coupling to intracellular Ca^{2+} mobilization is also involved in the inhibition of adipocyte differentiation [22,23]. To examine the possible involvement of EP4 receptor signaling in the inhibition of adipocyte differentiation and to discriminate it from FP-mediated inhibition, we investigated the effects of an EP4 antagonist on PGE_2- or fluprostenol-elicited inhibition of differentiation (Fig. 5). PGE_2 dose-dependently decreased cellular TG accumulation with an EC_{50} value of 10^{-8} M in the absence of an EP4 antagonist. The EP4 antagonist AE3–208 decreased this inhibition and shifted the dose-dependent curve rightward by two orders. In contrast, fluprostenol also showed an inhibitory effect on differentiation in our system, but this inhibition was insensitive to AE3–208 treatment. These results suggest that PGE_2 exhibits an inhibitory effect on differentiation via EP4 receptor activation, which is unrelated to FP receptor stimulation.

An EP4 antagonist promotes adipocyte differentiation

Endogenous prostanoids have been suggested to be involved in the inhibition of adipocyte differentiation, since COX inhibitors such as indomethacin display enhancing effects on differentiation [24,25]. We therefore investigated whether the differentiation-promoting effects of indomethacin (10 μM) are mimicked by an EP4 antagonist (AE3–208, 1 μM). As reported previously, indomethacin significantly promoted the differentiation of adipocytes as revealed by the increased TG content (Fig. 6). AE3–208 also showed an enhancing

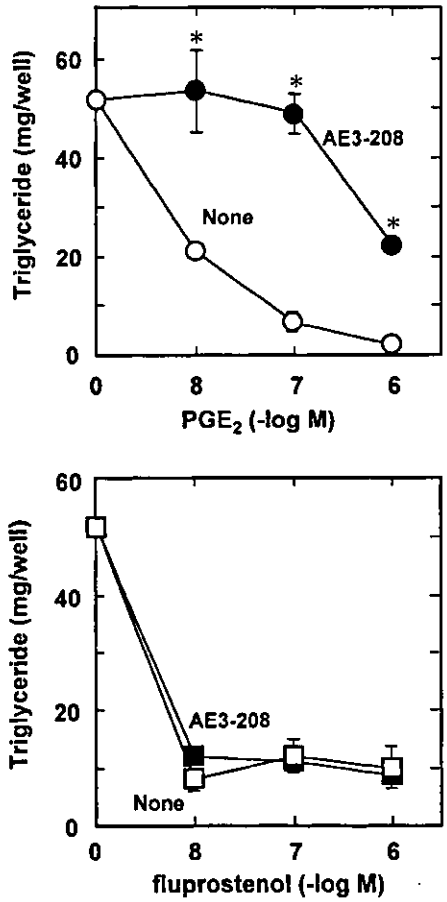

Fig. 5. Effects of an EP4 antagonist on PGE_2- or fluprostenol-elicited inhibition of 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail supplemented with the indicated concentrations of PGE_2 or fluprostenol in the presence (AE3–208) or absence of 1 μM AE3–208 (none). On day 8 of the differentiation program, cells were suspended in 2-propanol, and their triglyceride contents were determined as described in Materials and methods. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

effect on differentiation. These results suggest that endogenously synthesized PGE_2 has a suppressive role in 3T3-L1 differentiation by acting on the EP4 receptor.

Discussion

PGs have long been thought to contribute to fat cell development, but the role of PGs in the regulation of adipocyte differentiation is complex and has remained unclear [4]. One of the reasons for its complexity is that different classes of PGs exert opposing effects on differentiation. For instance, both PGI_2 and PGE_2, the two PGs predominantly synthesized by fat cells, appear to have opposing effects on early adipogenesis; PGI_2 promotes adipocyte differentiation [26,27], whereas PGE_2

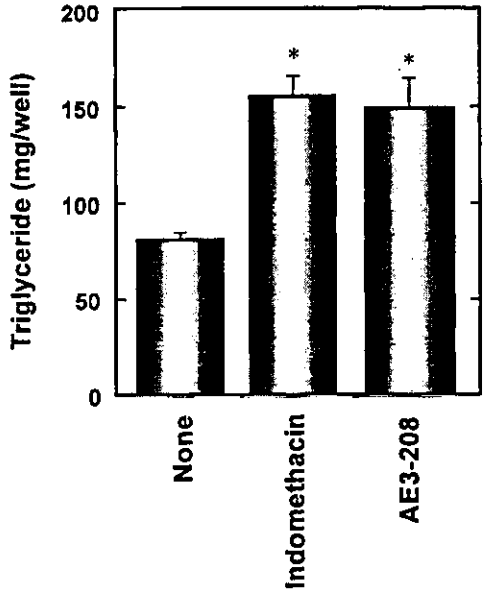

Fig. 6. Effects of indomethacin and an EP4 antagonist on 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with indomethacin (10 μM) or AE3–208 (1 μM). On day 8 of the differentiation program, cells were suspended in 2-propanol, and their triglyceride contents were determined as described in Materials and methods. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

inhibits differentiation. PGI_2 exerts its action by acting on the prostanoid IP receptor, while PGE_2 exhibits its versatile actions through its binding to four kinds of EP receptor subtypes with quite different signaling pathways. We hypothesized that the complex role of PGs in adipogenesis may be explained by the expression of multiple EP receptor subtypes in preadipocytes. Indeed, Borglum et al. [17] previously reported the existence of mRNAs for several kinds of PG receptors and EP receptor subtypes in Ob1771 preadipocytes. However, which PGE receptor(s) are functionally involved in the regulation of adipocyte differentiation remained poorly understood. In this study, we focused on identifying the receptor subtype(s) involved in the inhibition of adipocyte differentiation by PGE_2, and demonstrated that EP4 is the receptor responsible for this inhibition. The EP4 receptor is coupled to stimulation of adenylyl cyclase. Indeed, we demonstrated that an EP4 agonist as well as PGE_2 elicits an increase in intracellular cAMP levels in 3T3-L1 preadipocytes. We also showed that the cAMP analogue dibutyryl cAMP inhibits adipocyte differentiation as reported previously [24,28]. These results suggest that EP4 inhibits adipocyte differentiation in a cAMP-dependent manner.

It has been reported that exogenously added arachidonic acid inhibits 3T3-L1 differentiation [29]. When cells were treated with COX inhibitors, the inhibition of

differentiation was reversed, indicating that a COX-derived PG is necessary for arachidonic acid to inhibit differentiation. Both PGE_2 and $PGF_{2\alpha}$ are able to substitute for arachidonic acid when added to the differentiation cocktail [24]. Casimir et al. [22,23] demonstrated that $PGF_{2\alpha}$ as well as an FP-agonist inhibits differentiation more effectively than PGE_2, and thus they proposed that $PGF_{2\alpha}$ is the main PG involved in the inhibition of differentiation and that PGE_2 may only exert its inhibitory action by cross-reacting to the FP receptor. However, the current study demonstrated that the effect of PGE_2 but not that of $PGF_{2\alpha}$ is reversed by an EP4 antagonist. These results suggest that inhibition of adipocyte differentiation by PGE_2 is mediated by the EP4 receptor and is distinct from $PGF_{2\alpha}$ inhibition. Because arachidonic acid is converted more efficiently into PGE_2 than $PGF_{2\alpha}$ in 3T3-L1 cells [30], and because suppression by arachidonic acid is reversed by an inhibitor for protein kinase A [28], the inhibitory effect of arachidonic acid may be mediated through the PGE_2–EP4 pathway.

One of the interesting findings in this study is differentiation-dependent induction of EP1 gene expression. It was reported that the EP1 mRNA is expressed in mature adipocytes isolated from mouse adipose tissue [17]. Since EP1 has been shown to couple with phospholipase C and intracellular Ca^{2+} mobilization, EP1 might also inhibit differentiation as FP receptor does. However, an EP1-agonist failed to elicit significant inhibitory effects on adipocyte differentiation. Differentiation-dependent gene expression suggested that EP1 may be involved in some function of mature adipocytes. Indeed, it was reported that PGE_2 increases the calcium concentration and oxygen consumption in rat brown adipose tissue [31]. PGE_2 may stimulate metabolism of brown adipose tissue via EP1 receptor.

The current study demonstrated that the differentiation-enhancing effects of indomethacin can be mimicked by an EP4 antagonist. This result indicates that EP4 negatively regulates the standard differentiation process of 3T3-L1 cells. Yan et al. [32] reported that both a COX-1- and COX-2-inhibitor enhances differentiation of 3T3-L1 cells, indicating that both COX isozymes participate in the negative regulation of adipogenesis. Interestingly, Yan et al. also demonstrated that COX-2 inhibitors, but not a COX-1 inhibitor, reversed TNF-α-induced inhibition of differentiation. A similar modulating effect of COX-2 has been shown in adiponectin signaling [33]. Involvement of the EP4 signaling pathway in these systems is an interesting issue to examine in the future.

Acknowledgments

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Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice

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Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice

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[1]*Department of Applied Pharmacology, Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto, 607-8414;* [2]*Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Yoshida, Sakyo-ku, Kyoto, 606-8501; and* [3]*Department of Cellular Pharmacology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai, Japan 980-8575*

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Nakamura, Eiji, Takashi Kataoka, Kazuharu Furutani, Keisuke Jimbo, Takeshi Aihara, Satoshi Tanaka, Atsushi Ichikawa, Hiroshi Ohtsu, and Susumu Okabe. Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 287: G1053–G1061, 2004. First published July 22, 2004; doi:10.1152/ajpgi.00353.2003.—Histamine plays an important role in the regulation of gastric acid secretion; however, its role in maintenance of gastric morphology remains unclear. To clarify the necessity of histamine for gastric mucosal development and maintenance, we evaluated two different kinds of mice that lacked either mast cells (one of the gastric histamine-producing cell types) or histidine decarboxylase (HDC; a histamine-synthesizing enzyme). Measurements of stomach weight, intragastric pH, mucosal histamine levels, as well as serum gastrin and albumin levels were performed in mice. Gastric mucosal appearance was examined by immunohistochemical techniques. Although gastric mucosal histamine levels in mast cell-deficient mice were half of those observed in the wild-type mice, intragastric pH, serum gastrin levels, and gastric morphology at 12 mo were unchanged compared with the wild-type mice. In contrast, HDC-deficient mice possessed no detectable gastric histamine, but did exhibit hypergastrinemia, as well as marked increases in intragastric pH and stomach weight compared with the wild-type mice. Histological analysis revealed that 9-mo-old HDC-deficient mice demonstrated hyperplasia in the oxyntic glandular base region, as well as increased numbers of parietal and enterochromaffin-like cells. These results indicate that enterochromaffin-like cell-derived histamine is potentially involved in gastric mucosal morphology regulation.

enterochromaffin-like cell; parietal cell; hypergastrinemia

GASTRIC MUCOSA CONSISTS OF numerous blind tubular units containing various cell types (28). All gastric mucosal epithelial cells are known to originate from common progenitor cells in the proliferative zone of the isthmus. Some cells migrate upward, becoming mucous-secreting surface epithelial cells, whereas other cells migrate toward the base of the gland, differentiating into parietal, chief, or endocrine cells (18). Maintenance of gastric mucosal integrity and structure is regulated by a variety of endocrine- and paracrine-mediating factors.

Histamine is synthesized from histidine by histidine decarboxylase (HDC) and is stored in mast cells, enterochromaffin-like (ECL) cells, and enteric nerve fibers in the oxyntic mucosa of rodent stomachs (25, 46). It is well established that histamine plays a pivotal role in gastric acid secretion (1, 47); however, its role in the regulation of gastric mucosal proliferation and differentiation essentially remains uncharacterized. To clarify such a role, previous reports (16, 39, 40) have used pharmacological inhibitors of histamine pathways, such as histamine receptor (H_1R, H_2R, and H_3R) antagonists and α-fluoromethylhistidine (α-FMH), an irreversible inhibitor of HDC (4, 5, 10). Nonetheless, incomplete elimination of histamine action by these inhibitors could potentially confuse analysis of the exact role of histamine for regulation of gastric mucosal morphology.

Recently, HDC-deficient (HDC-KO) mice have been generated by gene-targeting methods (45, 52). As expected, these mice exhibited no de novo gastric mucosal histamine synthesis (52). Using 8- to 12-wk-old HDC-KO mice, we reported that gastric mucosal morphology was similar to the wild-type (WT) mice (52), although HDC-KO mice showed hypoacidity and hypergastrinemia (24, 52). In this study, we examined the role of histamine in the regulation of gastric mucosal morphology in HDC-KO mice longer term, from 1 to 9 mo. In addition, the present study characterized the specific role of ECL cell-derived histamine by comparing the difference between HDC-KO mice and mast cell-deficient mice.

MATERIALS AND METHODS

Animals. Six-week-old male mast cell-deficient WBB6F$_1$-*W*/*W*^{v} (*W*/*W*^{v}) and congenic normal WBB6F$_1$-+/+ (+/+) mice were purchased (Japan SLC, Shizuoka, Japan). The two mice strains were independently maintained until an age of 3 or 12 mo. Male HDC-KO as well as the WT littermate mice were generated on a mixed genetic 129/Sv times ICR background, and raised with regular diet, and independently maintained until an age of 1, 3, 6, or 9 mo (24, 45, 52). All of the mice were given standard pellets (CE-2; CLEA, Tokyo, Japan). Mice were deprived of food for 21 h and water for 2 h before each experiment. Plasma and tissue samples from pairs of age-matched WT and mutant mice were compared. Animal maintenance and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of Kyoto Pharmaceutical University.

Measurement of serum gastrin and albumin levels, as well as intragastric pH. Blood collected from mice was centrifuged at 6,000 *g* for 15 min to obtain serum samples. Serum gastrin levels were determined by a radioimmunoassay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan) and expressed as picograms of gastrin per

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milliliter of serum. Serum albumin levels were determined by a bromcresol green assay (6, 21) and expressed as grams of albumin per deciliter of serum. Stomachs removed from mice were incised along the greater curvature, and intragastric pH was measured by directly placing a pH meter on the fundic mucosa.

Determination of gastric mucosal histamine levels. Each sample for histamine quantification was collected according to a previously reported method (29). In brief, each stomach was rinsed with PBS containing 10^{-6} M semicarbazide hydrochloride, weighed, and homogenized in 0.01 M PBS. The homogenates were diluted 1:10 with phosphate buffer and heated in boiling water for 10 min to release bound histamine. The homogenates were then centrifuged at 3,300 *g* for 20 min, and the resulting supernatants were used for measurements. Quantification of sample histamine levels was performed with a histamine enzyme immunoassay kit (Immunotech, Marseilles, France).

Measurement of gastric mucosal protein and DNA levels. To measure gastric mucosal protein levels, the entire glandular stomach of each mouse was homogenized with 1 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 1 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 0.1% aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The homogenates were centrifuged at 10,000 *g* for 30 min at 4°C. The protein levels for each supernatant were measured with a protein quantification kit (Bio-Rad, Hercules, CA) and expressed as milligrams of protein per stomach. To quantify DNA levels, high molecular weight DNA was first isolated by digestion of the stomach with proteinase K, followed by phenol-chloroform extraction. The concentration of DNA solubilized in water was then quantified from the absorbance at 260 nm and the DNA levels in gastric mucosa was calculated and expressed as micrograms of DNA per stomach. The ratios of the protein levels or the DNA levels between HDC-KO and WT mice (KO/WT ratio) were calculated and compared each ratio to evaluate whether it is hyperplasia (increased DNA levels) or hypertrophy (increased cell volume, but not DNA levels).

Treatment. To evaluate the role played by hypergastrinemia in gastric oxyntic mucosal hyperplasia induced in HDC-KO mice, (R)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) (YM022) urea from Yamanouchi Pharmaceutical (Tokyo, Japan) (31, 41) was administered orally for 2 mo to 1-mo-old HDC-KO mice. YM022 represents a selective cholecystokinin type 2 receptor (CCK_2R) antagonist; a dose of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ was used, because this dose inhibited gastrin-17-stimulated gastric acid secretion in mice by >80% in our preliminary experiments. Omeprazole was administered orally at a dose of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 mo to 1-mo-old WT mice to induce hypergastrinemia and allow comparison with HDC-KO mice in terms of hyperplasia. Both drugs were suspended in 0.5% hydroxypropylcellulose solution and were administered once daily at a volume of 5 ml/kg body wt.

Histochemical analysis. For hematoxylin and eosin staining, as well as immunonhistochemical analysis, of the gastric mucosa, the stomach samples were fixed with Carnoy's fixative overnight, embedded in paraffin wax, and then sectioned at a slice thickness of 4 μm. Paraffin sections stained with hematoxylin and eosin or periodic acid Schiff were used for determination of gastric mucosal thickness. Under a microscope, well-orientated regions spanning from the gastric base to the oxyntic mucosal surface were selected for measurement. The mucosal thickness was taken as the average of measurements in four different visual fields for each stomach with the use of a calibrated-eyepiece micrometer scale. Immunohistochemical analysis of parietal and endocrine cells was performed for each section with the use of the following avidin-biotin-peroxidase immunohistochemical technique. Parietal, D, and endocrine cells were detected with a murine monoclonal antibody for α-subunit of murine H^+-K^+-ATPase (Medical and Biological Laboratories, Nagoya, Japan), a rabbit antibody for somatostatin (DAKO, Carpinteria, CA), and a rabbit antibody for a peptide (359–389 amino acid) of rat chromogranin A (Cg A; Yanaihara Institute, Fujinomiya, Japan), respectively. In brief, endogenous peroxidase activity was blocked with a methanol solution containing 0.3% hydrogen peroxide. Sections were incubated with primary antibody for α subunit of H^+-K^+-ATPase or Cg A for 2 h at room temperature. Sections were then incubated with secondary biotinylated antibodies, followed by streptoavidin peroxidase. Finally, slides were developed with diaminobenzidine and counterstained with hematoxylin. Parietal and endocrine cell counts were quantified in samples in which mucosal glands were perpendicularly oriented to the mucosal surface. Cell counts for parietal cells positively immunoreactive for H^+-K^+-ATPase were determined in four different gland units for each stomach; results were expressed as the number of cells per gland unit. Cell counts for endocrine cells positively immunoreactive for Cg A or D cells positively reactive for somatostatin were determined in four different visual fields for each stomach; results were expressed as the average number of cells per visual field (0.25 mm^2). An average for each group of animals was also calculated.

Statistics. Data are expressed as means ± SE. Statistical differences were evaluated by using the Student's *t*-test and Dunnett's multiple comparison test, with a P value < 0.05 regarded as significant. Dunnett's test was performed after a significant ANOVA had been achieved.

RESULTS

Mast cell deficiency exerted no effect on gastric morphology. Deletion of mast cells (*W/W^v*) caused a significant decrease in gastric mucosal histamine levels by ~50% compared with +/+ mice (Fig. 1*A*). However, the reduction in histamine levels did not affect intragastric pH (Fig. 1*B*) or serum gastrin levels (Fig. 1*C*), which were found to be similar for both +/+ and *W/W^v* mice. In addition, histological analysis revealed that *W/W^v* mice failed to exhibit any morphological alterations in the gastric oxyntic mucosa, even 3 and 12 mo after birth (Fig. 1, *D–G*). Ulcer was observed only in the gastric antrum of *W/W^v* mice (data not shown), corresponding with the previous report by Shimada et al. (49).

Long-term histamine deficiency resulted in gastric mucosal hyperplasia, hypochlorydria, and hypergastrinemia in HDC-KO mice. HDC-KO mice developed without any obvious abnormality in general appearance, exhibiting body weight gain similar to WT mice for ≤9 mo after birth. As expected, HDC-KO mice possessed undetectable histamine in the stomach (Fig. 2*A*). It was of interest that intragastric pH in HDC-KO mice was significantly higher than that measured in WT mice at all time points examined (1, 3, 6, and 9 mo after birth; Fig. 2*B*). In addition, HDC-KO mice exhibited a marked age-dependent gastric weight gain compared with WT mice, which was first obvious 3 mo after birth (Fig. 2*C*). Moreover, HDC-KO mice serum gastrin levels remained markedly elevated at all time points examined (Fig. 2*D*). Serum albumin levels were similar for WT and HDC-KO mice 6 and 9 mo after birth (Fig. 2*E*). No correlation was observed between body weight and stomach weight in either the WT or HDC-KO mice.

To thoroughly ascertain the increased stomach weight observed in HDC-KO mice, gastric mucosal protein and DNA levels were compared for 6-mo-old WT and HDC-KO mice. Protein levels in HDC-KO mice were significantly higher than those of WT mice (17.47 ± 1.89 mg/stomach vs. 11.80 ± 0.74 mg/stomach; KO/WT, 1.48). DNA levels in HDC-KO mice were approximately two times higher than those of WT mice

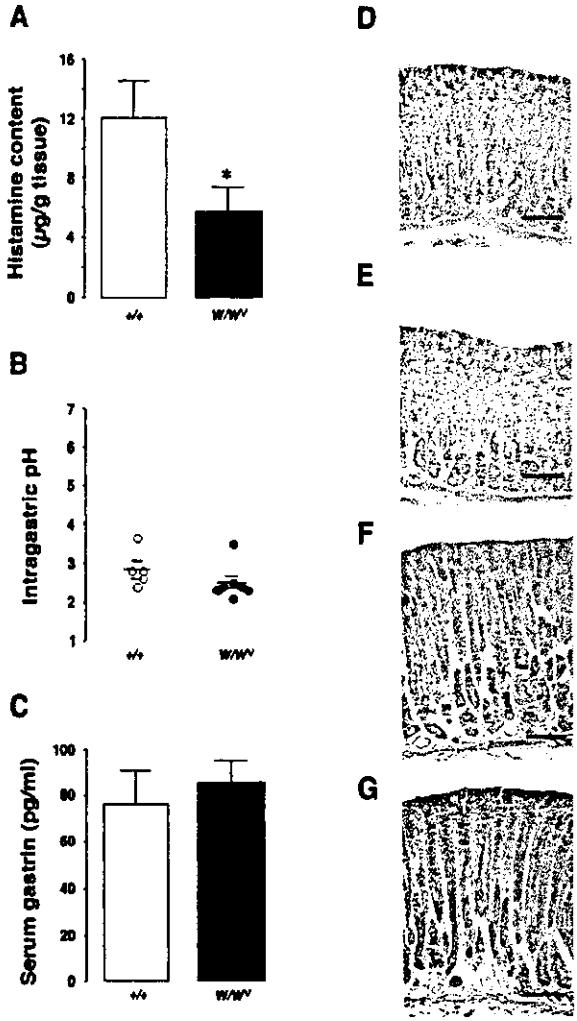

Fig. 1. Histamine levels (*A*), intragastric pH (*B*), and serum gastrin levels (*C*) in 3-mo-old wild-type (WT; +/+) and mast cell-deficient (*W/W*^{v}) mice. Values are means ± SE, n = 5–8. *Significant differences from WT mice with $P < 0.05$. *D–G*: histological comparison of gastric oxyntic mucosa in 3 (*D* and *E*)- and 12-mo-old (*F* and *G*) +/+ (*D* and *F*) and *W/W*^{v} (*E* and *G*) mice. Paraffin-embedded sections were prepared with hematoxylin and eosin staining (bar = 100 μm).

(982.1 ± 172.7 vs. 490.7 ± 49.9 μg/stomach; KO/WT, 2.00). These data indicate that the increase in HDC-KO mice stomach weight resulted from an increased number of gastric mucosal cells (hyperplasia), rather than an expanded cellular volume (hypertrophy).

Increased number of parietal and endocrine cells in HDC-KO mice. To identify the predominant cell types present in the hyperplastic mucosa of HDC-KO mice, histochemical analysis was performed. Six months after the birth of HDC-KO mice, their stomachs demonstrated a statistically significant increase in gastric mucosal thickness compared with WT mice. Interestingly, periodic acid Schiff staining revealed that the change in mucosal thickness was most prominent in the glandular basal region (WT, 389.0 ± 11.2 μm vs. HDC-KO, 570.0 ± 29.4 μm; Fig. 3, *A–C*, filled column). In contrast, no remarkable differences were observed in the pit region (WT, 74.8 ± 6.2 vs. HDC-KO, 74.5 ± 3.9 μm; Fig. 3, *A–C*, open column).

Time-course analysis revealed that gastric mucosal thickness in HDC-KO mice significantly increased after 1 mo compared with that of WT mice (Figs. 4*A*; 5, *A* and *B*; and 6, *A* and *B*), correlating with the increase in stomach weight (Fig. 2*C*). The hyperplastic changes were observed in the oxyntic mucosa but not in the antrum (Fig. 6, *A* and *B*). In addition, immunohistochemical analysis revealed that, in the oxyntic mucosa of HDC-KO mice, there were marked increases in the density of Cg A-positive endocrine cells (Figs. 4*B*; 5, *C* and *D*; and 6) and H^+-K^+-ATPase α-subunit-positive parietal cells (Figs. 4*C* and 5, *E* and *F*), compared with WT mice in a time-dependent manner. Cg A-positive endocrine cells in WT mice mainly distributed in the glandular basal region of the oxyntic mucosa (Fig. 6*A*). In contrast, in HDC-KO mice, distribution of the positive cells extended throughout the thickened mucosa (Fig. 6*B*). Interestingly, the size of parietal cells in HDC-KO mice was clearly smaller than that observed in WT mice, although no change in the size of the nucleus was noted (Fig. 5, *G* and *H*). In contrast, the density of somatostatin-positive D cell in gastric oxyntic mucosa was similar for HDC-KO and WT mice (data not shown). These results indicate that mucosal hyperplasia observed in HDC-KO mice resulted from increased numbers of Cg A-positive ECL and parietal cells.

Contribution of hypergastrinemia to gastric mucosal hyperplasia induced in HDC-KO mice. To examine the role played by hypergastrinemia for the hyperplastic changes observed in HDC-KO mice, pharmacological analysis was performed with omeprazole and YM022. Omeprazole treatment at a dose of 30 mg/kg once daily for 2 mo to 1-mo-old WT mice resulted in increases in serum gastrin levels (Fig. 7*A*), gastric mucosal thickness (Fig. 7*B*), and the number of Cg A-positive (Fig. 7*C*) and parietal cells (Fig. 7*D*), compared with vehicle-treated WT mice. In contrast, serum gastrin levels in vehicle-treated HDC-KO mice increased to similar levels observed in omeprazole-treated WT mice (Fig. 7*A*). Nonetheless, it was interesting to note that vehicle-treated HDC-KO mice exhibited more significant increases in mucosal thickness (Fig. 7*B*) and the number of Cg A-positive (Fig. 7*C*) and parietal cells (Fig. 7*D*) than omeprazole-treated WT mice. Treatment of HDC-KO mice with YM022 recovered the histological parameters to the levels in omeprazole-treated WT mice, but not to the levels in vehicle-treated WT mice. It should also be noted that YM022 augmented the increase in serum gastrin levels induced in HDC-KO mice.

DISCUSSION

The present study demonstrated that long-term histamine deficiency ≤9 mo attained with HDC deletion led to gastric mucosal morphological alteration with time. As a matter of fact, apparent oxyntic mucosal hyperplasia, i.e., increases in parietal and Cg A-positive endocrine cell counts, was observed in HDC-KO mice. Gastric mucosal thickening mainly resulted from changes that developed in the glandular base region of the gastric mucosa. In addition, gastric mucosal cells in HDC-KO

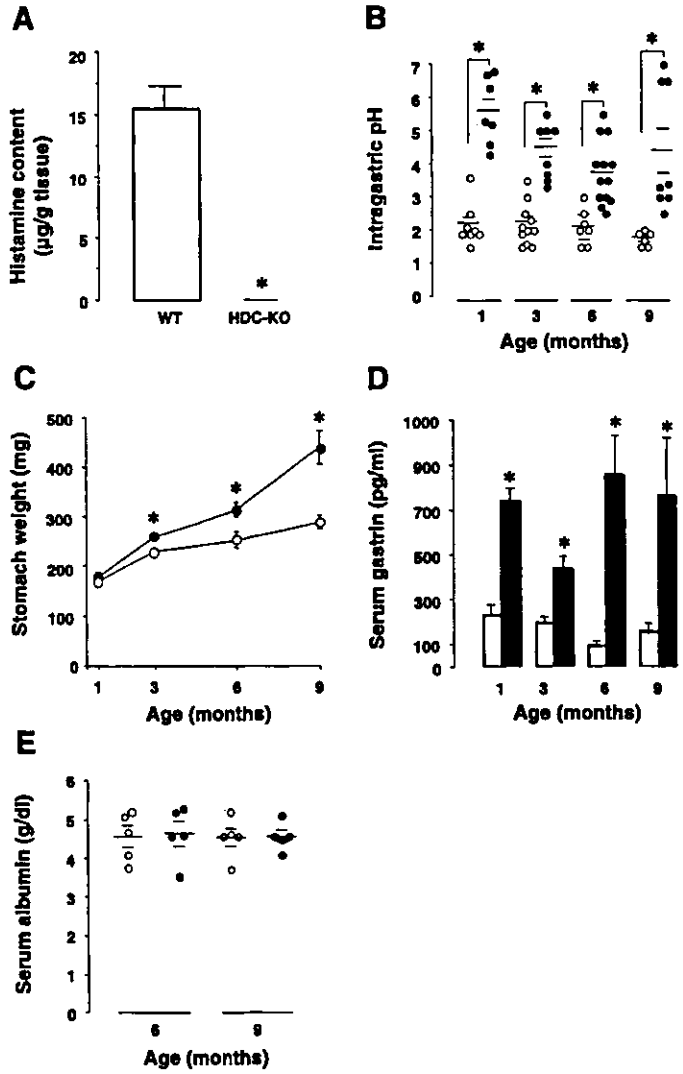

Fig. 2. *A*: histamine levels in 6-mo-old WT and histidine decarboxylase-deficient (HDC-KO) mice (*A*). Time course changes in intragastric pH (*B*), stomach weight (*C*), and serum gastrin (*D*) and albumin levels (*E*) in WT (open circle or column) and HDC-KO (closed circle or column) mice. Values are means ± SE, $n = 4$–13. *Significant differences from WT mice of the same age, with $P < 0.05$.

mice, especially parietal cells, were observed to be smaller in size than the cells in WT mice. Some of the above observations were consistent with the previous three different gene-targeted mice studies including HDC-KO (27), gastrin-deficient (26), and our H_2 receptor-deficient (H_2R-KO) mice (29, 44). Hunyady et al. (27) reported a increase in the number of parietal cells in HDC-KO mice >9 mo old. Both H_2R-KO and gastrin-deficient mice are reported to possess defects in ECL cell function, i.e., histamine production (26, 29, 44). H_2R-KO mice impair the entry of histamine signal from ECL cells into the parietal cells, and subsequently exhibited an increased stomach weight, enlarged gastric folds with cystic dilatation, hyperplasia of smaller-sized parietal and ECL cells, and hypergastrinemia (44). These results indicate that histamine is not essentially required for differentiation of progenitor stem cells to parietal, ECL, or other endocrine cells but is required to maintain normal gastric mucosal architecture and population with aging.

It is of note that, in addition to the pathological changes mentioned above, hypoalbuminemia was observed in H_2R-KO mice. This finding is considered the hallmark of Menetrier's disease (7, 33, 55) and is thought to result from albumin loss from cystic dilatation of the gastric mucosa (32, 48). Accordingly, we postulated that abnormalities in H_2R function lie at the heart of the pathogenesis of the disease (44). We expected similar pathological changes in HDC-KO mice, because we assumed that a lack of histamine (HDC-KO) might be similar to deletion of gastric mucosal H_2R (H_2R-KO). However, the present study demonstrated that there was no difference in serum albumin levels for HDC-KO and WT mice. In addition, gastric hyperplastic mucosa was much milder in HDC-KO