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

Yoshinori Kawazoe,[1] Satoshi Tanaka,[2] and Motonari Uesugi[1,*]

[1]The Verna and Marrs McLean Department of Biochemistry and Molecular Biology
Baylor College of Medicine
Houston, Texas 77030

[2]Department of Physiological Chemistry
Graduate School of Pharmaceutical Sciences
Kyoto University
Sakyo-ku
606-8501 Kyoto
Japan

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),

*Correspondence: muesugi@bcm.tmc.edu

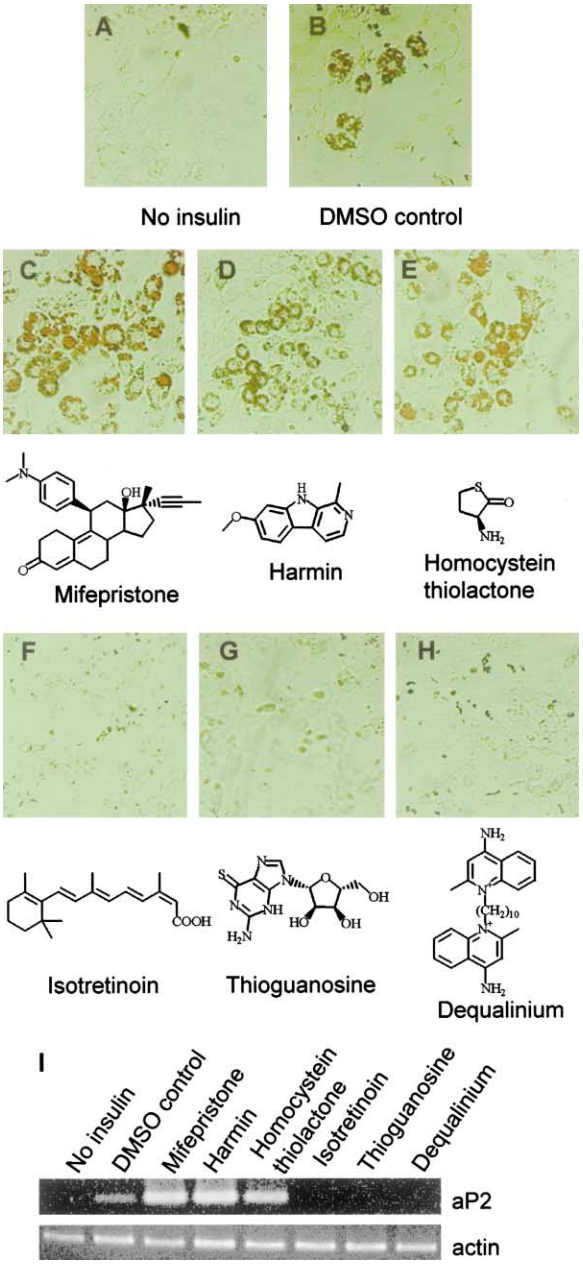

Figure 1. Effects of 880 Bioactive Compounds on Insulin-Induced Adipogenesis of 3T3-L1 Cells

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.

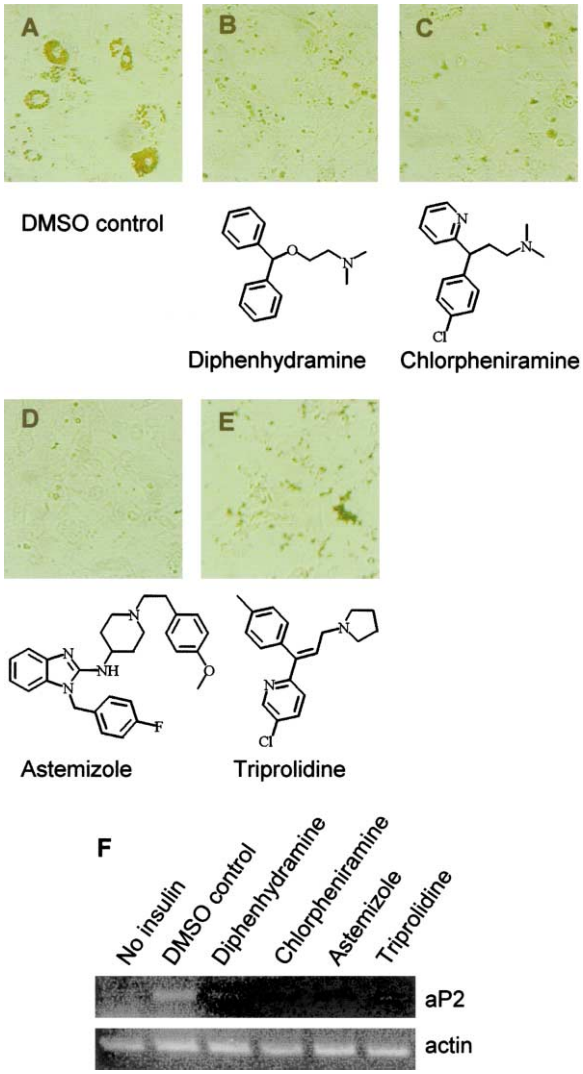

Figure 2. Inhibition of Adipogenic Differentiation by Histamine Blockers

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.

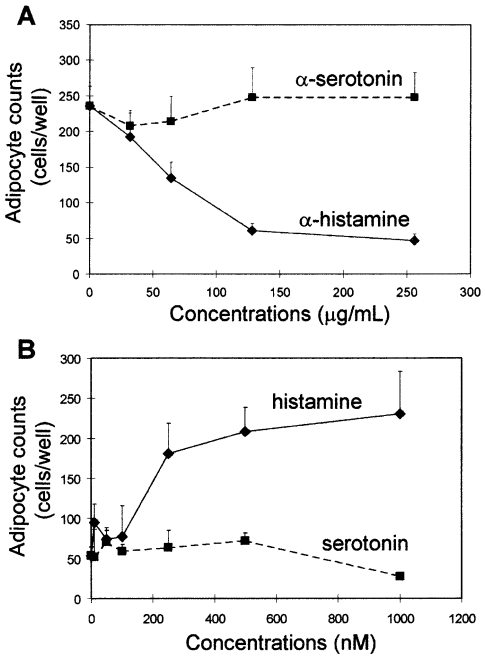

Figure 3. Inhibition of Adipogenic Differentiation by an Anti-Histamine Antibody

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.

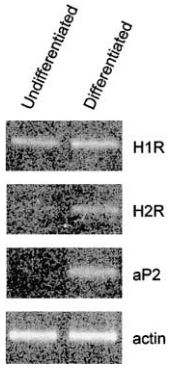

Figure 4. Expression Levels of the Histamine H1 and H2 Receptors in Undifferentiated and Fully Differentiated 3T3-L1 Cells

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.

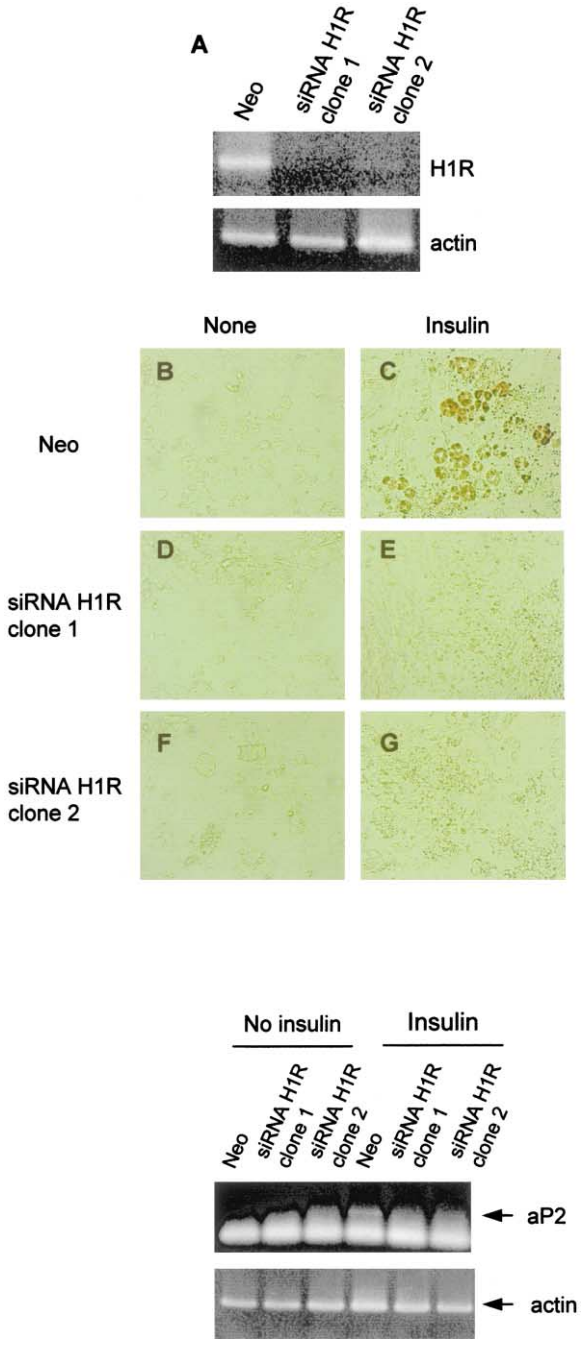

Figure 5. Inhibition of Insulin-Induced Adipogenesis by siRNA Knockdown of the Histamine H1 Receptor

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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