Studies on the Novel Molecular Mechanism of Lipolysis and Adipose Tissue Inflammation

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Abstract

Obesity, caused by the imbalance of energy storage and expenditure, has become a worldwide health problem and is accompanied by low-grade chronic systemic inflammation, which can lead to type 2 diabetes and increased risk of chronic diseases. The systemic inflammation is caused, at least in part, by adipose tissue inflammation. The adipose tissue plays a central role in the regulation of whole-body energy homeostasis. Homeostasis in adipose tissue is a balance of adipogenesis and lipolysis. In obese state, adipogenesis exceeds lipolysis, and the adipose tissue undergoes dynamic remodeling by increasing number and size of adipocytes. At the pathological stage, hypoxia develops as adipocytes rapidly enlarge with limited angiogenesis. Microenvironmental hypoxia stimulates M1-stage macrophages infiltration and inflammatory cytokines production that is strongly associated with systemic inflammation and insulin resistance. Thus, repressing adipose tissue from pathological expansion by stimulating lipolysis and preventing adipose tissue inflammation is critically important for the improvement of obesity and prevention of following health problems. To provide new insights of improving obesity, I focused on the pathological expansion of adipose tissue in this study.

First, I examined the lipolytic effects of green tea catechins, a well-known food ingredient, with their mechanisms in the first chapter. I revealed that green tea catechins stimulate lipolysis in the presence of norepinephrine via a PKA-dependent pathway in adipocytes. The results suggest that dietary tea catechins, especially when combined with physical activity, might have the potential to stimulate body fat reduction.

Second, I focused on an intestinal hormone GIP, which is known as an incretin that stimulate insulin secretion in response to postprandial blood glucose. Since genetic ablation of GIPR attenuates obesity induced by a high-fat diet and further improves insulin sensitivity in mice, GIP has been suggested to be related in obesity development,

whose mechanism has not been fully understood. I clarified that chronic GIP increase induces adipose tissue inflammation and disclose a novel factor, HIF-1α, as an underlying contributor to GIP-induced adipose inflammation via its upregulation of GIPR. This makes HIF-1α a therapeutic target for the development of GIP-based treatments for obesity and type 2 diabetes.

Taken together, these results from my studies shed the light on green tea catechins and GIP on prevention and improvement of obesity. Daily consumption of green tea catechins and intake of meal that decreases GIP secretion could be useful not only for prevention of body fat accumulation but also for reduction of obesity.

Abbreviations

aP2	adipocyte protein 2
ATGL	adipose triglyceride lipase;
AMPK	adenosine-monophosphate-activated protein kinase
BSA	bovine serum albumin
CEBPα	CCAAT/enhancer-binding protein α
CEBP δ	CCAAT/enhancer binding protein δ
COMT	catechol-O-methyltransferase
DMEM	Dulbecco's modified Eagle's medium
EGCG	(-)-epigallocatechin gallate
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
GIP	glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GTCs	green tea catechins
HIF-1α	hypoxia-inducible factor-1α
GLUT1	glucose transporter 1
HSL	hormone-sensitive lipase
IL-1β	interleukin-1β
IL-6	interleukin-6
ITT	insulin tolerance test
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MEK	MAPK/ERK kinase
NE	Norepinephrine

PAI1	plasminogen activator inhibitor 1
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PPAR γ	peroxisome proliferator-activated receptor γ
qRT-PCR	quantitative reverse transcription -PCR
SEM	standard error of the mean
siRNA	small interfering RNA
SREBP-1c	sterol regulatory element binding protein-1c
TNFα	tumor necrosis factor α
VMA	vanillylmandelic acid
VEGFa	vascular endothelial growth factor a
36B4	acidic ribosomal protein P0

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General Introduction

Obesity and adipose tissue remodeling

Obesity, caused by the imbalance of energy storage and expenditure, has become a worldwide health problem and is accompanied by low-grade chronic systemic inflammation. This systemic inflammation is frequently associated with the development of insulin resistance, which can lead to type 2 diabetes and increased risk of chronic diseases such as cardiovascular disease and cancers [1, 2]. The systemic inflammation is caused, at least in part, by adipose tissue inflammation.

The adipose tissue plays a central role in the regulation of whole-body energy homeostasis. Homeostasis in adipose tissue is a balance of adipogenesis and lipolysis. In obese state, adipogenesis exceeds lipolysis, and the adipose tissue undergoes dynamic remodeling, including quantitative (hyperplasia) and qualitative (hypertrophy) alterations of adipocytes. Hyperplasia is linked to beneficial phenomena, such as increased adiponectin, decreased basal fatty acids release, pro-inflammatory cytokine release, and improved insulin sensitivity. Hypertrophy of adipose tissue can be divided into metabolically healthy and pathological stages. At the healthy stage, expansion of adipose tissue accompanied with enough angiogenesis and appropriate remodeling of the extracellular matrix with minimum inflammation. In contrast, at the pathological stage, rapid enlargement of the adipocytes with limited angiogenesis affect the microenvironment of expanded adipose tissues and ensuing hypoxia, which in turn can cause the induction of a fibrotic program. Ultimately, M1-stage macrophages infiltrate, which further stimulates the production of inflammatory cytokines that is strongly associated with systemic inflammation and insulin resistance.

Thus, repressing adipose tissue from pathological expansion by stimulating lipolysis and preventing adipose tissue inflammation is critically important for the

improvement of obesity and prevention of following health problems.

Green tea catechins

Food ingredients have been widely studied for their efficacy to prevent obesity, and several plant components have been shown to exhibit anti-obesity effects [3]. Tea, which is the most popularly consumed beverage aside from water, has been consumed worldwide since ancient times to maintain and improve health. Tea is particularly rich in polyphenols, including catechins, theaflavins and thearubigins, which are thought to contribute to the health benefits of tea. Depending upon the level of fermentation, tea can be categorized into three types: green (unfermented), oolong (partially fermented) and black (highly to fully fermented).

Green tea is most abundant of tea catechins include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG), which have been shown to be epimerized to (-)-catechin (C), (-)-gallocatechin (GC), (-)-catechin gallate (CG) and (-)-gallocatechin gallate (GCG) during heat treatment. Various studies have provided evidence that green tea catechins (GTCs) are the strong biologically active agents, which exhibit various health-promoting effects including antioxidant effects, anti-microbial effects, anti-viral effects, neuro-protective effects, anti-cancer effects, and anti-obesity effects.

GTCs show anti-obesity effects in a direct and indirect manner. Murase *et al* have shown that GTCs activate adenosine-monophosphate-activated protein kinase (AMPK), and thereby increase fatty acid β-oxidation in liver and skeletal muscle [4], which is considered to be the indirect mechanism of its anti-obesity effect. GTCs also directly decrease adipogenesis in adipocytes *in vitro* [5-8] by suppressing preadipocyte

proliferation and differentiation, inhibiting the lipogenic enzymes in mature adipocytes, and inducing adipocyte apoptosis. These findings strongly support the effectiveness of GTCs in prevention of obesity. In addition, ingestion of GTCs not only suppresses body fat accumulation [9, 10], but also reduces body fat mass in humans [11, 12], especially dietary GTCs enhance exercise-induced fat loss in humans [13]. These findings stimulated my interest in investigating the lipolytic potential of dietary GTCs, which has not been fully understood.

Glucose-dependent insulinotropic polypeptide

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino-acid hormone secreted from intestinal enteroendocrine K cells in response to dietary fat or glucose [14, 15]. GIP is recognized as an incretin hormone that potentiates glucose-stimulated insulin secretion from pancreatic β cells via its specific receptor (GIPR) and regulates postprandial glucose homeostasis. GIPR exists in multiple organs and has been shown to have extra-pancreatic effects such as stimulating progenitor cell proliferation on the brain, increasing bone formation, and stimulating lipogenesis in adipose tissue [16] (Fig.1).

GIP stimulates lipogenesis and contributes to energy storage as fat in adipocytes through GIPR [17]. Of curious, a high-fat diet dramatically increases GIP secretion [18], and the blood concentration of GIP is elevated in obesity [19-21]. Genetic ablation of GIPR attenuates obesity induced by a high-fat diet and further improves insulin sensitivity in mice [22]. Blood GIP levels are also elevated in diabetic patients because its insulinotropic activity is blunted [19, 21]. Moreover, studies have shown that GIP stimulates proinflammatory cytokine secretion and impairs insulin signaling in

adipocytes [23, 24].

On the basis of these findings, I hypothesized that increased GIP signaling in obesity and type 2 diabetes plays an important role in the pathogenesis of insulin resistance by inducing adipose tissue inflammation. To date, however, no direct evidence has been available on the role of GIP signaling in adipose tissue inflammation or insulin resistance *in vivo*. To elucidate the role and the mechanism of GIP induced adipose tissue inflammation may provide a new strategy to improve obesity and type 2 diabetes.

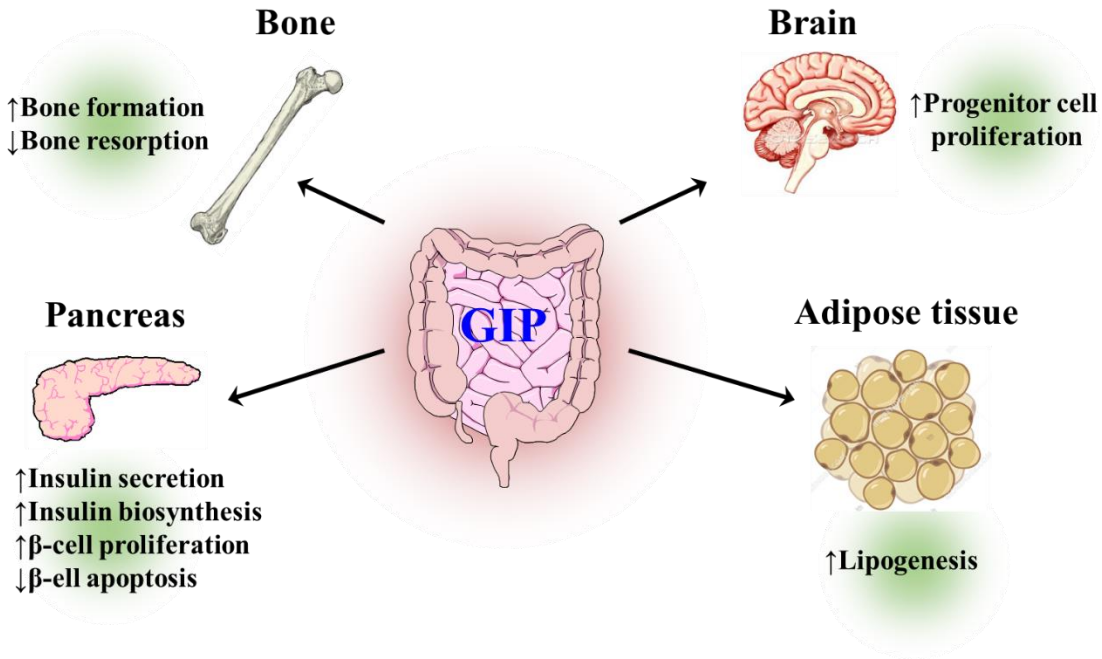


Fig. 1 GIP actions in peripheral tissues

Objective of my studies

To provide new insights of improving obesity and metabolic syndrome, I focused on the pathological expansion of adipose tissue in this study, examined the lipolytic effects of tea catechins with their mechanisms in the first chapter, and revealed the physiological functions of GIP signaling in the pathogenesis of adipose tissue inflammation in the second chapter (Fig.2).

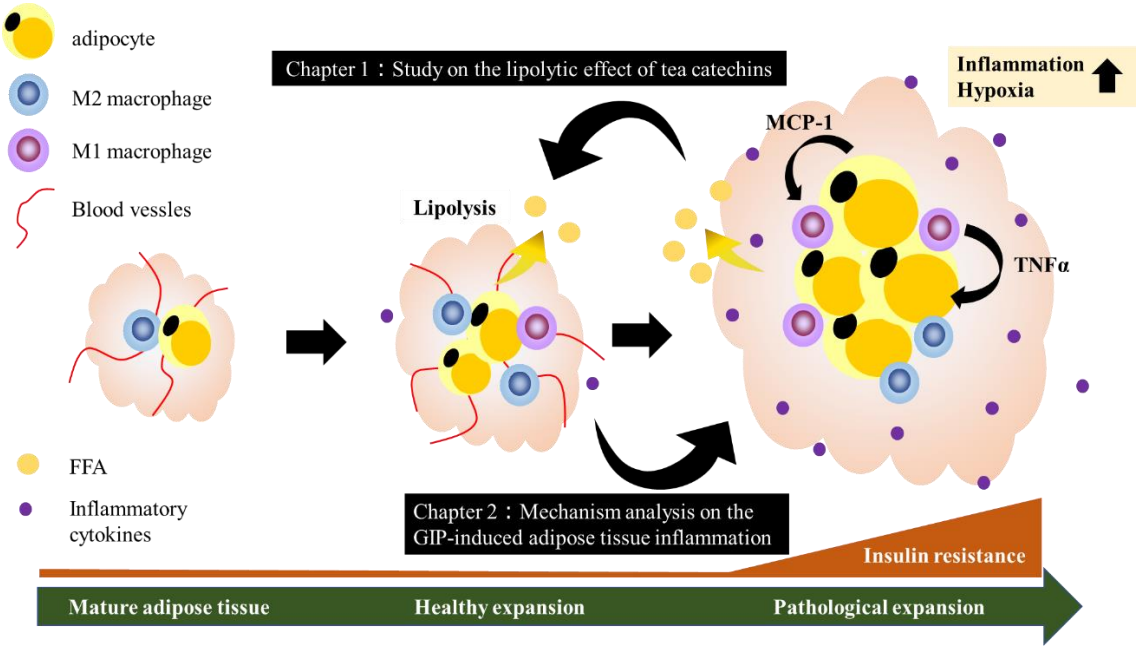


Fig. 2 Adipose tissue expansion and the concept of this study

Chapter1: Green tea catechins enhance norepinephrine-induced lipolysis via a protein kinase A-dependent pathway in adipocytes

Abstract

Green tea catechins have been shown to attenuate obesity in animals and humans. The catechins activate adenosine monophosphate-activated protein kinase (AMPK), and thereby increase fatty acid oxidation either in liver and skeletal muscle. Green tea catechins have also been shown to reduce body fat in humans. However, the effect of the catechins on lipolysis in adipose tissue has not been fully understood. The aim of this study was to clarify the effect of green tea catechins on lipolysis in adipocytes and to elucidate the underlying mechanism. Differentiated mouse adipocyte cell line (3T3-L1) was stimulated with green tea catechins in the presence or absence of norepinephrine. Glycerol and free fatty acids in the media were measured. Phosphorylation of hormone-sensitive lipase (HSL) was determined by Western blotting, and the mRNA expression levels of HSL, adipose triglyceride lipase (ATGL), and perilipin were determined by quantitative RT-PCR. The cells were treated with inhibitors of protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), or mitogen-activated protein kinase (MAPK) to determine the responsible pathway. Treatment of 3T3-L1 adipocytes with green tea catechins increased the level of glycerol and free fatty acids released into the media in the presence, but not absence, of norepinephrine, and increased the level of phosphorylated HSL in the cells. The catechins also increased mRNA and protein levels of HSL and ATGL. PKA inhibitor (H89) attenuated the catechin-induced increase in glycerol release and HSL phosphorylation. The results demonstrate that green tea catechins enhance lipolysis in the presence of norepinephrine via a PKA-dependent pathway in 3T3-L1 adipocytes, providing a potential mechanism by which green tea catechins could reduce body fat.

Introduction

Green tea catechins (GTCs) exhibit various health-promoting effects including an anti-obesity effect in animals [25] and humans [9, 10]. Murase *et al* have shown that GTCs activate adenosine-monophosphate-activated protein kinase (AMPK), and thereby increase fatty acid β-oxidation either in liver and skeletal muscle [4], which is considered to be the fundamental mechanism of its anti-obesity effect.

GTCs directly affect adipocytes *in vitro* [5-8]. Homeostasis in adipose tissue is a balance of adipogenesis and lipolysis. GTCs, particularly (-)-epigallocatechin gallate (EGCG), have been shown to decrease adipogenesis in 3T3-L1 adipocytes [5, 6]. EGCG prevents adipocyte differentiation by inhibiting the lipogenic enzymes peroxisome proliferator activator receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (CEBPα) [7]. EGCG also induces adipocyte apoptosis and preadipocyte proliferation, thereby preventing triglyceride accumulation in a dose-dependent manner [8].

Ingestion of GTCs not only suppresses body fat accumulation [9, 10], but also reduces body fat mass in humans [11, 12]. Furthermore, dietary GTCs enhance exercise-induced fat loss in humans [13]. These findings stimulated my interest in investigating the lipolytic potential of dietary GTCs.

The activity of three lipolytic enzymes, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase play a critical role in lipolysis in adipose tissues [26]. Catecholamines, epinephrine, and norepinephrine (NE), acting through β-adrenoceptors, increase cAMP production, thereby activating protein kinase A (PKA) and finally leading to phosphorylation of HSL (at Ser563, Ser659, and Ser660) and lipolysis [27-29]. In addition, activation of one or more of the PKC, PKG, and the MAPK pathway is involved in adipocyte lipolysis [30] (Fig. 3). Whereas EGCG

has been shown to increase lipolytic gene expression *in vitro* [31] and *in vivo* [32], knowledge of the effect of tea catechins on lipolysis is limited.

The aim of this study was to clarify the effect of green tea catechins on lipolysis and to elucidate the underlying mechanism in adipocytes. Since dietary green tea catechins enhance exercise-induced fat loss in humans [13], and NE production is increased during exercise [33, 34], I also examined the effect of the catechins on NE-induced lipolysis in 3T3-L1 adipocytes.

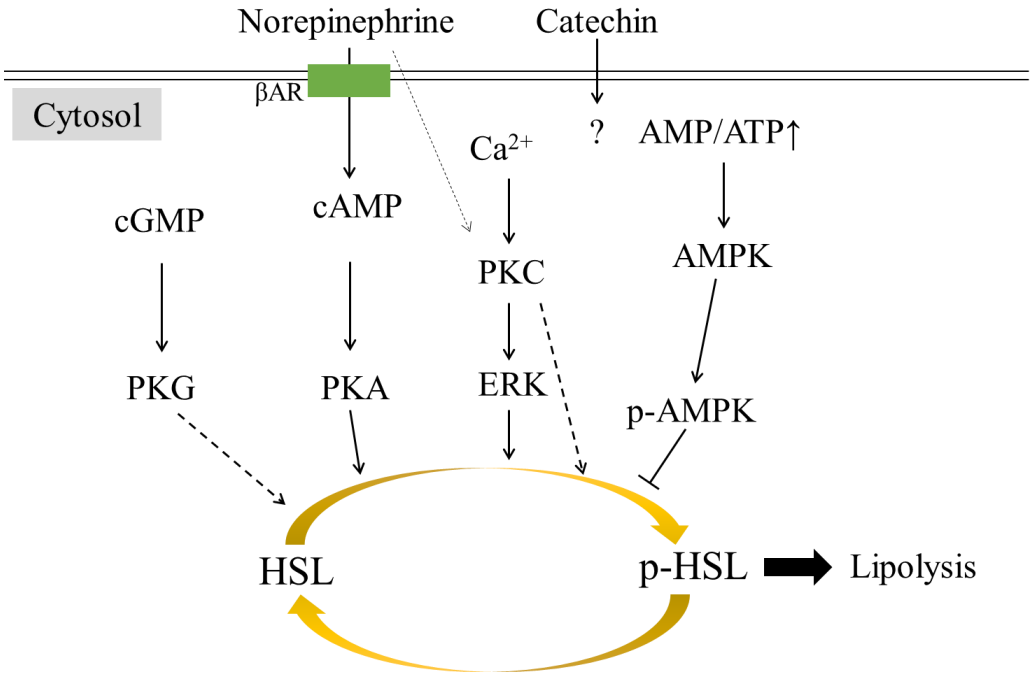


Fig. 3 Signal cascade of adipocyte lipolysis

Materials and Methods

GTCs were prepared and their composition analyzed as described previously [25]. The catechins comprised epigallocatechin gallate (38.2%), epigallocatechin (30.2%), epicatechin gallate (10.7%), epicatechin (7.9%), gallocatechin (6.8%), catechin (2.9%), gallocatechin gallate (2.0%), and others (1.3%).

Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co., LCC., Tokyo, Japan) containing 10% fetal bovine serum and penicillin-streptomycin (Life Technologies Japan Ltd., Tokyo, Japan), and incubated at 5% CO_2 in air at 37 °C. The preadipocytes were differentiated by treatment with insulin, dexamethasone, and 3-isobutyl-1-methyl-xanthine as described by Chen *et al* [35]. Cells on days 10–12 after the induction of differentiation were used for all experiments.

Treatment of the cells

After an overnight incubation in DMEM supplemented with 0.5% BSA, 3T3-L1 cells were treated with GTCs in the presence or absence of NE (0.1 or 1 μM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 6h or 24 h. In the protein kinase inhibitor experiments, the cells were treated with either H89 (PKA inhibitor; 5–50 μM; Cell Signaling Technology Japan K.K., Tokyo, Japan), Gö 6983 (PKC inhibitor; 1 μM; Cayman Chemical Company, Ann Arbor, MI), Rp-8-pCPT-cGMP (PKG inhibitor; 10 μM, Enzo Life Sciences, Inc., Farmingdale, NY), U0126 (MAPK inhibitor; 10 μM; Cell Signaling Technology), PD98059 (MAPK inhibitor; 20 μM; Cayman) for 2 h before the

GTC treatment. Isoproterenol (Ipt) (SigmaeAldrich) 10 mM was used as the positive control for inducing lipolysis in each experiment.

Lipolysis assay

Glycerol and free fatty acids (FFA) in the cell culture media were measured colorimetrically by using a glycerol assay kit (Sigma-Aldrich) and a NEFA C-test (Wako Pure Chemical Industries, Ltd.), respectively.

Quantitative RT-PCR

Total RNA was extracted by using a RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). Reverse transcription was performed by using a High-Capacity cDNA Kit with random primers on a Veriti 96-well Thermal Cycler (Life Technologies Japan Ltd.). Quantitative RT-PCR (qRT-PCR) was performed by using a TaqMan probe (Life Technologies Japan Ltd.) in an ABI ViiA 7 Real-Time PCR System (Life Technologies). All data were normalized to the transcript levels of the gene encoding acidic ribosomal protein P0 (36B4).

TaqMan probes used in the study are listed as below:

HSL: Mm00495359_m1, ATGL: Mm00503040_m1, Perilipin: Mm00558672_m1, 36B4:Mm00725448_s1

Western blotting

3T3-L1 adipocytes were lysed with CelLytic M (Sigma-Aldrich). Protein concentrations were measured by BCA Protein Assay (Thermo Fisher Scientific K.K., Yokohama, Japan). Ten micrograms of each sample was subjected to electrophoresis in a 4%–15% Criterion

gel (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were then transferred to polyvinylidene fluoride membranes and incubated with primary antibodies, followed by anti-rabbit or anti-mouse IgG, horseradish peroxidase-linked secondary antibody (Cell Signaling Technology Japan K.K.). Signals were detected by using the ECL Prime Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan) and a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc). Primary antibodies against HSL (#4107), p-HSL (Ser563, #4139), ATGL (#2138), and β-Actin (#4967) were all purchased from Cell Signaling Technology Japan K.K.

Statistics

Data were expressed as means ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used for comparisons between multiple groups. The threshold for significance was $P < 0.05$. All statistical analyses for this study were performed in Prism 8 (GraphPad Software Inc.)

Results

GTCs stimulates lipolysis in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with GTCs alone did not significantly change the level of glycerol (Fig. 4A) or FFA (Fig. 4B) in the media. NE alone (0.1 or 1.0 μM) increased the glycerol (Fig. 4A) and FFA (Fig. 4B) concentrations in a dose-dependent manner. GTCs significantly enhanced the NE-induced glycerol (Fig. 4A) and FFA (Fig. 4B) release into the media in a dose-dependent manner.

GTCs increases HSL phosphorylation in 3T3-L1 adipocytes

Treatment of the cells with GTCs alone did not change the level of phosphorylated HSL (p-HSL), total HSL protein, or ATGL protein (Fig. 5A). NE alone increased the level of p-HSL, but not that of total HSL or ATGL protein (Fig. 5B). GTCs enhanced the NE-induced increase in p-HSL in a dose-dependent manner, but did not change the total HSL or ATGL protein level (Fig. 5B).

GTCs increases lipolysis related genes and HSL protein expression in 3T3-L1 adipocytes

Treatment of the cells with GTCs alone significantly increased the mRNA expression

levels of HSL, ATGL, and perilipin (Fig. 6A) in a dose-dependent manner; the trend of increased ATGL mRNA expression was observed for all GTC concentrations tested (ie., 2.3, 11.5, and 23 μM), but was statistically significant for 11.5 μM GTC only. Long-term (24 h) treatment with GTCs also increased HSL protein level (Fig. 6B) dose-dependently.

H89, a PKA inhibitor, decreases GTC-induced lipolysis

Glycerol release from the cells in the media was significantly increased by GTC in the presence of NE (0.1 μM) (Figs. 7A and 7B). The GTC-induced glycerol release (in the presence of NE) was significantly decreased by H89 but not by other protein kinase inhibitors examined (Fig. 7A). H89 decreased the GTC-induced glycerol release (in the presence of NE) in a dose-dependent manner (Fig. 7B).

GTCs (23 μM) increased p-HSL protein, but not total HSL or ATGL proteins in the presence of NE (0.1 μM) (Fig. 7C). H89 suppressed the GTC-induced HSL protein phosphorylation in a dose-dependent manner, whereas it did not change total HSL or ATGL protein levels (Fig. 7C).

Discussion

This study has three major findings. First, GTCs enhanced NE-induced lipolysis in 3T3-L1 adipocytes. Second, GTCs increased NE-induced HSL phosphorylation. Finally, the GTC-enhanced lipolysis and HSL-phosphorylation in the presence of NE were inhibited by the PKA inhibitor H89, indicating that these effects were mediated by a PKA-dependent pathway.

In this study, GTCs alone increased expression of genes encoding lipolytic proteins (i.e., HSL, ATGL, and perilipin) in adipocytes. These results are consistent with those reported by Lee *et al*. [31, 32], who showed that EGCG increases HSL mRNA expression both in 3T3-L1 adipocytes and in mice. Phosphorylation of HSL protein activates and translocates the enzyme to the surface of lipid droplets, and where it hydrolyzes triglycerides [26-30]. Most interestingly, here I demonstrated that GTCs directly promoted phosphorylaton (i.e., activation) of HSL protein and increased lipolysis in the presence of NE in adipocytes.

My study provides evidence that GTC-enhanced lipolysis and HSL phosphorylation are mediated by a PKA-dependent pathway. PKA activation is a classical pathway for lipolysis regulation. NE increases cAMP production, which results in cAMP-dependent PKA activation, which in turn leads to HSL phosphorylation [27-29]. Two

enzymes phosphodiesterase (PDE) and catechol-O-methyltransferase (COMT) may be involved in this NE-PKA dependent lipolysis induced by GTCs (Fig. 8).

PDE hydrolyzes intracellular cAMP and inhibits PKA activation [36]. Therefore, PDE inhibition is likely to potentiate adipocyte lipolysis by maintaining the intracellular cAMP level. Plant flavonoids, such as quercetin, fisetin, luteolin, and genistein inhibit phosphodiesterase 3 and thereby induce lipolysis in rat adipocytes [37-39]. In my study, GTCs enhanced HSL phosphorylation only in the presence of NE. Therefore, one possible explanation is that GTCs enhanced NE-induced HSL phosphorylation by inhibiting PDE activity. However, Kuppusamy and Das showed that catechins stimulated PDE activity in rat adipocytes (EC_{50} = 130 to 240 μM) [37]. Since I examined much lower catechin concentrations than those in their study, further investigations are required to clarify the involvement of PDE inhibition by GTCs in the enhancement of NE-induced lipolysis.

COMT inhibition is the other possible mechanism that explains GTCs enhanced lipolysis in the presence of NE. COMT degrades catecholamines including NE into vanillylmandelic acid (VMA). EGCG and EGC have been shown to inhibit COMT activity at a very low concentration (EC_{50} ≒ 0.2 μM). Thus, it is very likely that GTCs inhibited NE degradation and enhanced NE-induced lipolysis, which should be further studied.

Although I showed that the PKA pathway is responsible for enhanced lipolysis by GTCs, my results also implicate the contribution of other signaling pathways. Whereas PKA inhibition by H89 (50 μM) diminished most of the HSL phosphorylation (Fig. 7C), lipolysis was not blocked completely (Fig. 7B). Since inhibitors of PKC, PKG, and MAPK did not affect GTC-induced lipolysis in this study, the activation of lipolysis by GTCs might be regulated by increased levels of HSL mRNA (Fig. 6A) and protein (Fig. 6B). However, further study is needed to clarify the mechanisms underlying the lipolytic activity of GTCs.

A potential weakness of the current study is that it is an *in vitro* study, and cannot directly predict *in vivo* results. Although several *in vivo* studies have examined the lipolytic activity of GTCs, further studies are required to examine the lipolytic activity of GTCs and determine whether lipolytic gene expression and direct activation of HSL by GTCs enhances lipid degradation in response to β-adrenergic signals, such as physical exercise and stress exposure. Lee *et al* showed that expression of genes encoding lipolytic proteins such as HSL and ATGL was increased in mice fed with 0.2% or 0.5% EGCG [32]. Murase *et al*. [4] showed that oral administration of EGCG (200 mg/kg body weight) to mice increased fat oxidation, partly by activating AMPK activity in the liver. Previous studies showed that catechin consumption combined with exercise additively stimulated

fat oxidation and prevented body fat accumulation in mice [40, 41]. Dietary GTCs have been shown to enhance exercise-induced fat loss in humans [13]. Murase *et al*. previously demonstrated that catechin consumption plus exercise improves endurance capacity by promoting fat oxidation in mice [42]. Since physical exercise increases NE and induces lipolysis in adipocytes [33, 34, 43], a daily intake of GTCs may enhance exercise-induced fatty acid release from body fat, and increase blood free fatty acids as energy substrate.

In conclusion, this study demonstrates that green tea catechins stimulate lipolysis in the presence of NE via a PKA-dependent pathway in 3T3-L1 adipocytes. The results suggest that dietary tea catechins, especially when combined with physical activity, might have the potential to stimulate body fat reduction. Daily consumption of green tea catechins could be useful not only for prevention of body fat accumulation but also for reduction of obesity.

Figures

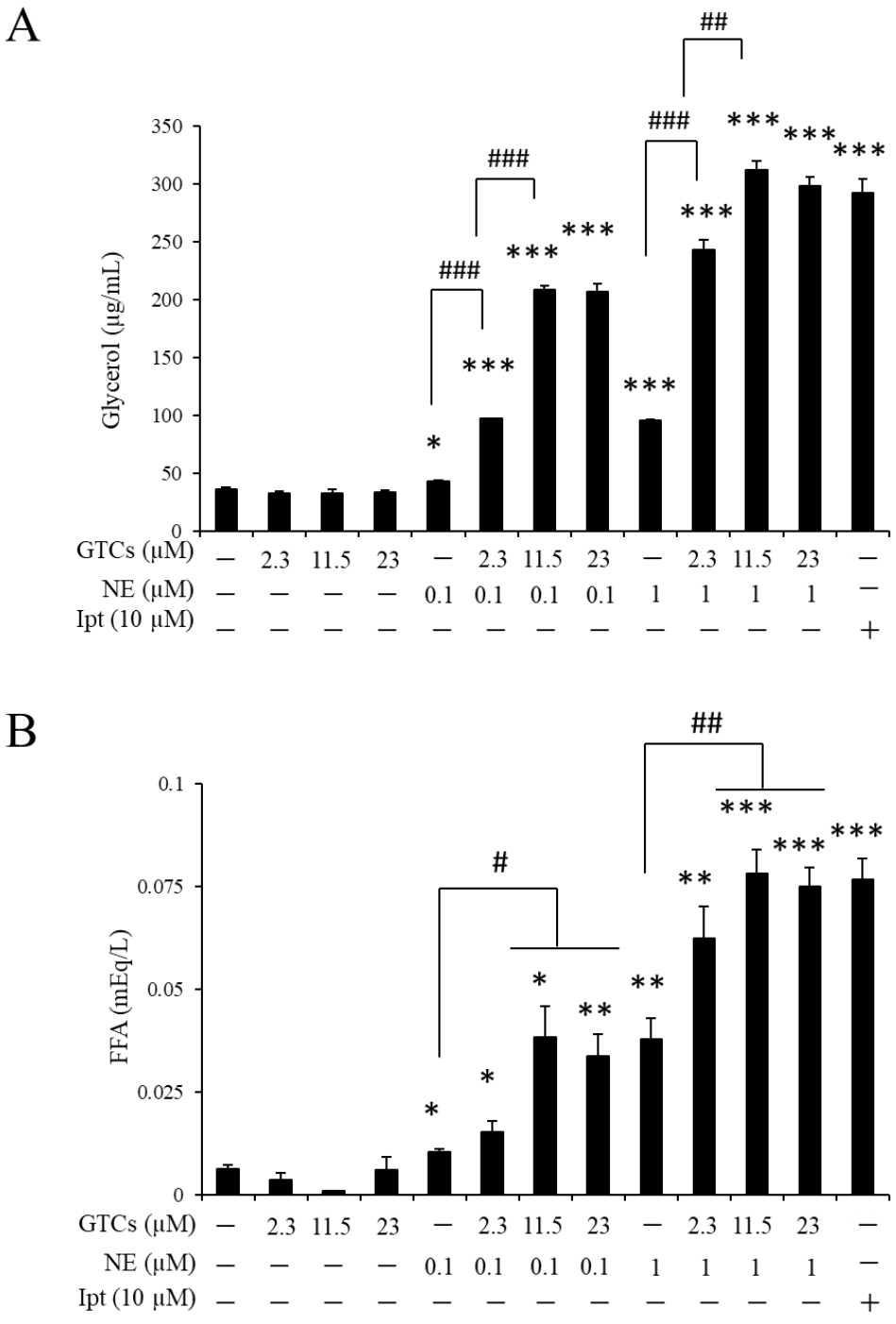


Fig. 4 Effect of GTCs on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with GTCs in the presence or absence of norepinephrine (NE)

(0.1 or 1 μM) in DMEM supplemented with 0.5% BSA. After 24-h treatment, the levels of glycerol (A) and free fatty acids (FFA) (B) in the media were determined. Isoproterenol (Ipt) (10 μM) was used as the positive control. Data are presented as means ± SEM (n = 3). * $P < 0.05$, * $P < 0.01$, *** $P < 0.001$ vs. GTCs (-) NE (-); ## $P < 0.01$, ### $P < 0.001$ (ANOVA with Tukey's multiple comparison test).

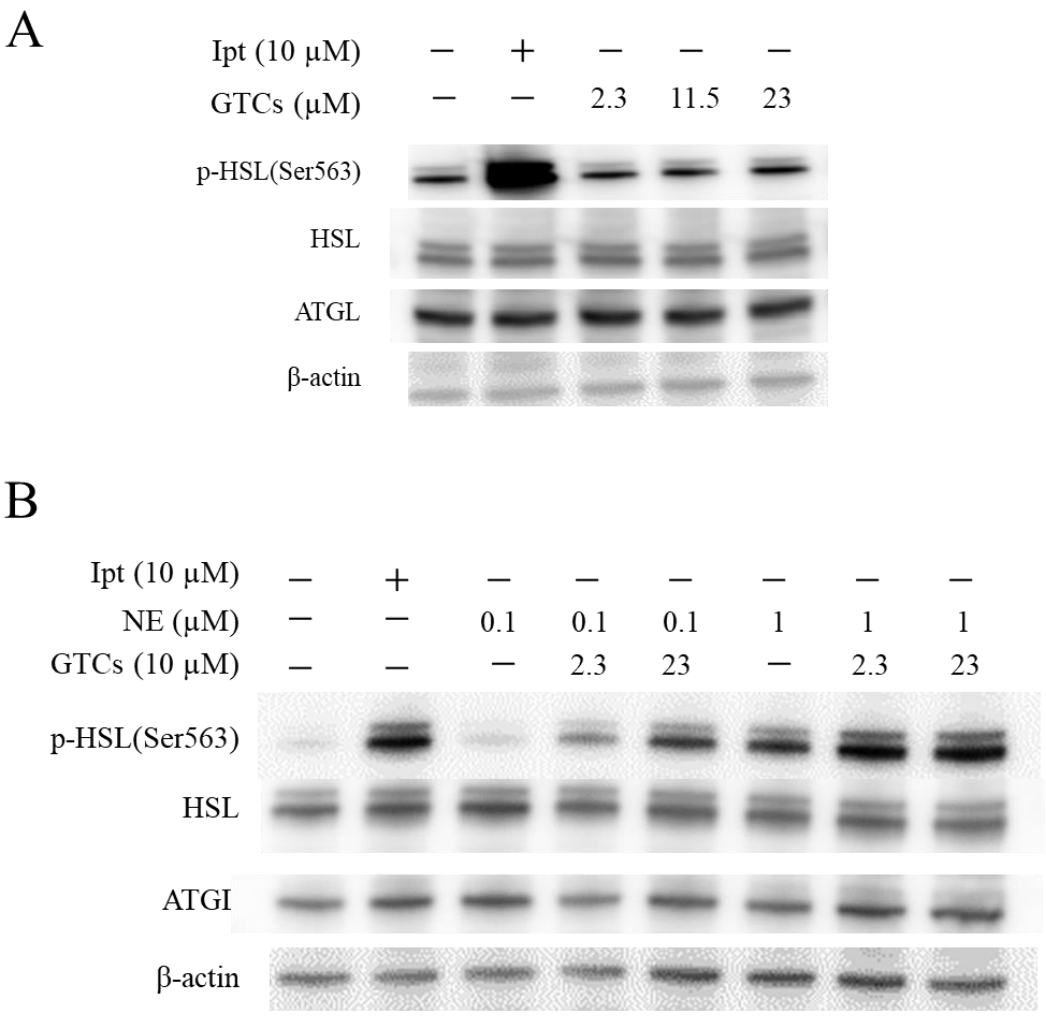


Fig. 5 Effect of GTCs on HSL phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated in DMEM supplemented with 0.5% BSA for 6 h, and then treated with GTCs in the absence (A) or presence (B) of norepinephrine (NE) (0.1 or 1 μM) for 30 min. The cells were then lysed and subjected to Western blot analysis. Protein levels of total HSL, phospho-HSL (Ser563), ATGL, and internal control β-actin were visualized. Isoproterenol (Ipt) (10 μM) was used as the positive control.

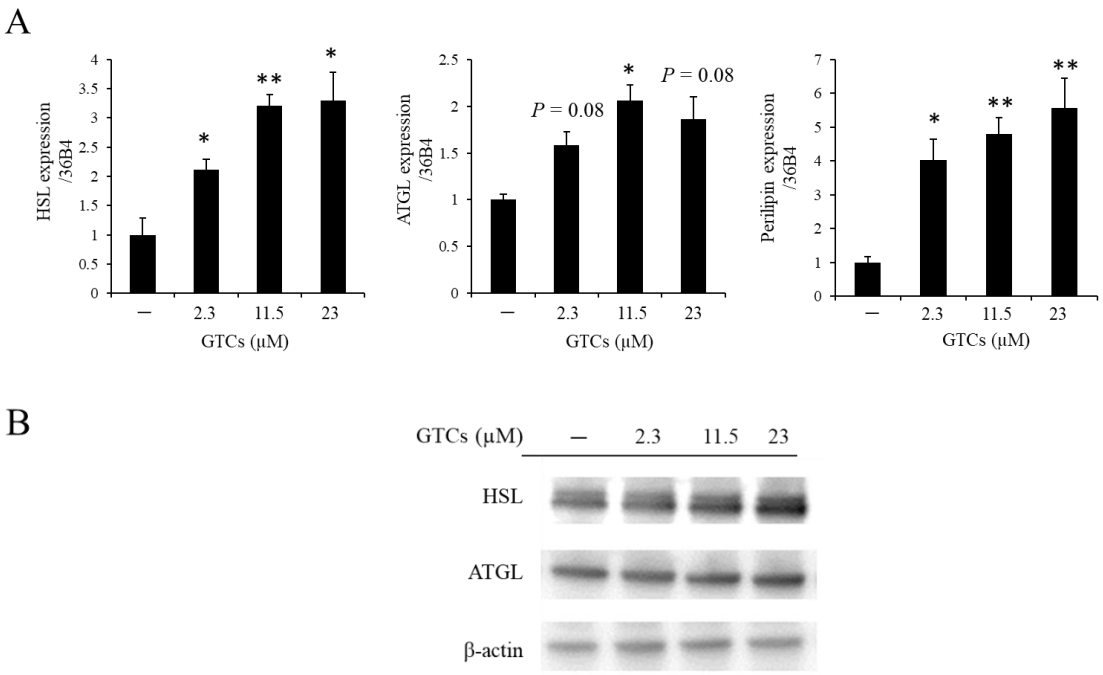


Fig. 6 Effect of GTCs on lipolytic enzyme expression in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with GTCs in DMEM supplemented with 0.5% BSA. After 24-h treatment, total RNA was isolated and the mRNA expression levels of HSL, ATGL, and perilipin were determined by qRT-PCR and normalized to the 36B4 expression level (A). Data are presented as means ± SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, vs. GTCs (-) (ANOVA with Tukey's multiple comparison test). Differentiated 3T3-L1 adipocytes treated as described for (A) were lysed and subjected to Western blot analysis, and protein levels of total HSL, ATGL, and internal control β-actin were visualized (B). The images are representative of 3 independent experiments.

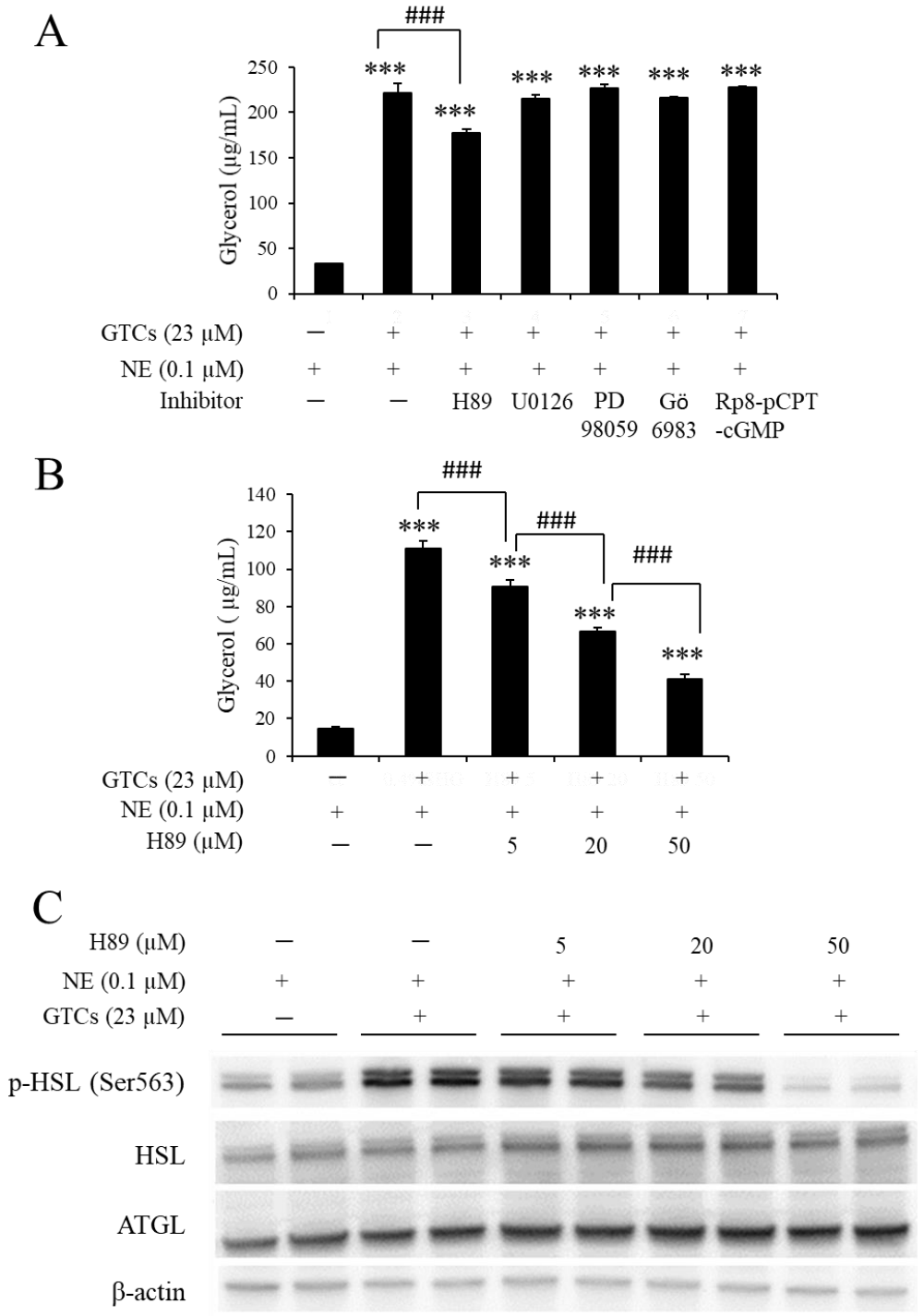


Fig. 7 Effect of protein kinase inhibitors on GTC-induced lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated for 24 h with H89 (PKA inhibitor, 5–50 μM) (A, B), U0126 (MAPK inhibitor, 10 μM), PD98059 (MAPK inhibitor,

20 μM), Gö 6983 (PKC inhibitor, 1 μM), or Rp-8-pCPT-cGMP (PKG inhibitor, 10 μM) (A) plus GTCs (23 μM) and (NE) (0.1 μM) in DMEM supplemented with 0.5% BSA, and then the level of glycerol released into the media was determined (A, B). Data are presented as means ± SEM (n = 3). *** $P < 0.001$ vs. GTCs (-), NE(+), H89 (-), ### $P <$ 0.001 (ANOVA with Tukey's multiple comparison test). In panel (C), differentiated 3T3-L1 adipocytes were incubated in DMEM supplemented with 0.5% BSA for 6 h. The cells were then treated with H89 (5–50 μM) in DMEM supplemented with 0.5% BSA for 2 h, followed by GTCs (23 μM) in the presence of NE (0.1 μM) and H89 (5–50 μM) for 30 min. The cells were lysed and subjected to Western blot analysis. Protein levels of total HSL, phospho-HSL (p-HSL Ser563), ATGL, and internal control β-actin were analyzed.

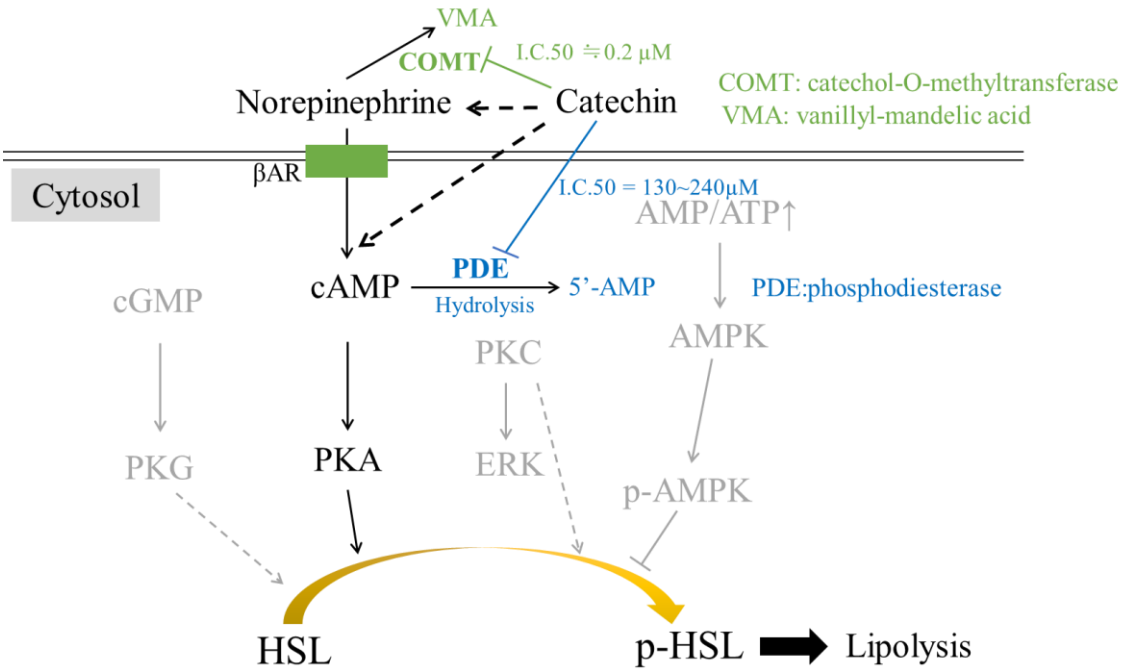


Fig. 8 Possible mechanism of catechin-induced PKA-dependent lipolysis

Chapter 2: Increased GIP signaling induces adipose inflammation via a HIF-1α–dependent pathway and impairs insulin sensitivity in mice

Abstract

Glucose-dependent insulinotropic polypeptide (GIP) is a gut hormone secreted in response to dietary fat and glucose. The blood GIP level is elevated in obesity and diabetes. GIP stimulates proinflammatory gene expression and impairs insulin sensitivity in cultured adipocytes. In obesity, hypoxia within adipose tissue can induce inflammation. The aims of this study were 1) to examine the proinflammatory effect of increased GIP signaling in adipose tissues *in vivo*, and 2) to clarify the association between GIP and hypoxic signaling in adipose tissue inflammation. I administered GIP intraperitoneally to misty (lean) and db/db (obese) mice and examined adipose tissue inflammation and insulin sensitivity. I also examined the effects of GIP and hypoxia on expression of the GIP receptor (GIPR) gene and proinflammatory genes in 3T3-L1 adipocytes. GIP administration increased monocyte chemoattractant protein 1 (MCP-1) expression and macrophage infiltration into adipose tissue and increased blood glucose in db/db mice. GIPR and hypoxia-inducible factor-1α (HIF-1α) expression were positively correlated in the adipose tissue in mice. GIPR expression increased dramatically in differentiated adipocytes. GIP treatment of adipocytes increased MCP-1 and interleukin 6 (IL-6) production. Adipocytes cultured either with RAW264 macrophages or under hypoxia expressed more GIPR and HIF-1α, and GIP treatment increased gene expression of

plasminogen activator inhibitor 1 and IL-6. HIF-1α gene silencing diminished both macrophage- and hypoxia-induced GIPR expression and GIP-induced IL-6 expression in adipocytes. Thus, increased GIP signaling plays a significant role in adipose tissue inflammation and thereby insulin resistance in obese mice, and HIF-1α may contribute to this process.

Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a gut hormone secreted from intestinal enteroendocrine cells in response to dietary fat or glucose [14, 15]. A high-fat diet dramatically increases GIP secretion [18]. The blood concentration of GIP is elevated in obesity [19-21]. GIP potentiates glucose-stimulated insulin secretion from pancreatic β cells via its specific receptor (GIPR) [44, 45]. GIPR is also located in adipose tissue [16] and contributes to energy storage as fat in adipocytes [17]. Genetic ablation of GIPR attenuates obesity induced by a high-fat diet and further improves insulin sensitivity in mice [22]. Blood GIP levels are also elevated in diabetic patients because its insulinotropic activity is blunted [19, 21]. More recently, studies have shown that GIP stimulates proinflammatory cytokine secretion and impairs insulin signaling in adipocytes [23, 24].

On the basis of these findings, I hypothesized that increased GIP signaling in obesity and type 2 diabetes plays an important role in the pathogenesis of insulin resistance by inducing adipose tissue inflammation. To date, however, no direct evidence has been available on the role of GIP signaling in adipose tissue inflammation or insulin resistance *in vivo*.

Adipocytes become hypertrophic in obesity, and hypoxic areas often develop

within the adipose tissue [46, 47]. Hypoxia can induce adipose tissue inflammation by inducing proinflammatory gene expression in adipocytes and macrophages [47, 48]. This effect is mediated through the activation of hypoxia-inducible factor (HIF)-1, a basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β. In hypoxic conditions, the HIF-1α protein level is dramatically increased through inhibition of its ubiquitination and proteasome-mediated degradation [49, 50]. HIF-1α is highly expressed in the adipose tissue of obese individuals and decreases after weight loss [51]. Hypoxia has been shown to impair insulin sensitivity in adipocytes [52]. Moreover, adipose tissue–specific HIF-1α deletion prevents the adipose tissue inflammation and insulin resistance induced by a high-fat diet in mice [53, 54].

Hypoxia and GIP signaling have each been associated with adipose tissue dysregulation [23, 24, 47, 53, 55, 56]. Hypoxia not only increases proinflammatory gene expression but also decreases adiponectin gene expression [47, 48]. Inhibition of GIP signaling increases adiponectin gene expression in adipose tissue and the blood adiponectin level in mice [56]. On the basis of the findings so far, I hypothesized that GIP signaling may interact with hypoxic signaling to cause adipose tissue inflammation. However, the association between GIP and hypoxic signaling in adipose tissue dysregulation is not clear.

Therefore, the aims of the present study were 1) to examine whether increased GIP signaling has a proinflammatory effect in adipose tissues *in vivo*, and 2) to clarify the association between GIP and hypoxic signaling in adipose tissue inflammation. Because hypoxia dramatically increased GIPR gene expression in adipocytes, I also investigated whether HIF-1α signaling is involved in the hypoxia-induced GIPR gene expression in adipocytes.

Materials and Methods

GIP treatment of mice

Male BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/J (db/db) and misty mice (6 to 10-wk old, Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed 3 per cage in a room with controlled temperature and relative humidity (23 ± 2.0 °C, 55% ± 10%) and a 12-h light-dark cycle. Six mice were assigned to each group in such a way that the average body weight of each group was equivalent. Mice were maintained for 1 or 4 wk on the designated diet (Table 1) and water was provided ad libitum.

During the experimental period, db/db mice and misty mice were intraperitoneally administered mouse GIP (AnaSpec, Inc., Fremont, CA; 10 nmol/kg body weight [BW]) twice daily (at 0900 h and 1700 h) for 1 wk, or mouse GIP (5 nmol/kg) once daily (at 0900 h) for 4 wk. For negative control mice, the same amount of saline (Otsuka Pharmaceutical, Co., Ltd, Tokyo, Japan) was administered.

Mice were anesthetized after 1 or 4 wk of GIP administration by the inhalation of isoflurane (Abbott Japan Co., LTD, Osaka, Japan). Blood samples were collected from the abdominal vein into capillary blood collection tubes (CAPIJECT with EDTA-2Na, Terumo Medical Co., Tokyo, Japan) and maintained on ice until plasma preparation. After centrifugation at 3500 × *g* for 15 min at 4 °C, plasma samples were stored at –80 °C until

analysis. The white adipose tissues (epididymal, mesenteric, perirenal, retroperitoneal, and inguinal) were removed and also stored at –80 °C. After 1 wk of GIP or saline administration, expression of inflammatory genes and macrophage infiltration were analyzed with quantitative reverse transcription (qRT)-PCR and immunohistochemistry, respectively.

All animal experiments were conducted in the Experimental Animal Facility of Kao Tochigi Institute. The Animal Care Committee of Kao Corporation approved the present study.

Insulin tolerance test

An insulin tolerance test (ITT) was performed after 3 wk of GIP or saline administration. The mice were fasted for 5 h before the ITT. Glucose levels in tail blood were measured with a blood glucose self-monitoring device (ACCU-CHEK Aviva, Roche Diagnostics Co., Tokyo, Japan) before and 30, 60, 90, and 120 min after an intraperitoneal injection of human insulin (db/db, 1 U/kg BW; misty, 0.5 U/kg BW).

Gene expression analysis by qRT-PCR

Total RNA was extracted by using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan)

or RNeasy Lipid Tissue Mini Kit (QIAGEN). Reverse transcription was performed by using a High-Capacity cDNA Kit with random primers on a Veriti 96-well Thermal Cycler (Life Technologies Japan Ltd., Tokyo, Japan). qRT-PCR was performed by using a TaqMan probe (Life Technologies) in an ABI ViiA 7 Real-Time PCR System (Life Technologies). All data were normalized to acidic ribosomal protein P0 (36B4) content.

TaqMan probes used in the study are listed as below:

MCP-1: Mm00441242_m1, IL-6: Mm00446190_m1, F4/80: Mm00802529_m1, GIPR: Mm01316344_m1, C/EBP α : Mm00514283_s1, C/EBP δ : Mm00786711_s1, aP2: Mm00445878_m1, PPAR γ : Mm01184322_m1, SREBP-1c: Mm00550338_m1, HIF-1 α : Mm00468869_m1, VEGFa: Mm01281449_m1, PAI1: Mm00435860_m1, Leptin: Mm00434759_m1, Adiponectin: Mm01343606_m1, GLUT1: Mm00441480_m1, 36B4:Mm00725448_s1

Immunohistochemical analysis

A small piece of retroperitoneal adipose tissue was fixed in 4% paraformaldehyde. After paraffin embedding, 3-μm tissue sections were deparaffinized and then treated with methanol containing 1% (v/v) H_2O_2. Antigen retrieval was performed by autoclaving for 120 min. Then the tissues were incubated with a rat anti-mouse F4/80 antibody

(MCA497R, 1:50; AbD Serotec, Kidlington, UK) overnight at 4 °C. Bound F4/80 antibodies were detected by using Histofine Simple Stain Mouse MAX-PO (Rat) (Nichirei Biosciences Inc., Tokyo, Japan). All light-field images were captured by using a BIOREBO BZ-9000 microscope (Keyence Corporation, Osaka, Japan).

Plasma Analysis

Blood glucose was determined with a Glucose CII test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma GIP was measured with a rat/mouse total GIP enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Tokyo Japan), and insulin was determined with a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

Monoculture of 3T3-L1 adipocytes and GIP treatment

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co., LCC., Tokyo, Japan) containing 10% FBS and penicillin-streptomycin (Life Technologies) and incubated in an atmosphere containing 5% CO_2 at 37 °C. Differentiation of 3T3-L1 preadipocytes into mature adipocytes was performed by using the same medium

containing 10 mg/L insulin (Sigma-Aldrich), 1 μmol/L dexamethasone (Sigma-Aldrich), and 0.5 mmol/L 3-isobutyl-1-methly-xanthine (Sigma-Aldrich) for 2 d. The medium was then replaced with DMEM containing 5 mg/L insulin for another 2 d, and with DMEM not containing insulin for a further 12 d. For experiments involving GIP treatment, mature 3T3-L1 adipocytes on day 6 were cultured in FBS-free DMEM for 6 h, followed by treatment with 10 or 100 nM GIP for 3 h.

Co-culture of adipocytes with RAW264 macrophages and GIP treatment

RAW264 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in DMEM containing 10% FBS and penicillin-streptomycin, and incubated in an atmosphere containing 5% CO_2 at 37 °C. Differentiated 3T3-L1 adipocytes were co-cultured with RAW264 cells in accordance with the procedure of Suganami *et al* [57]. Briefly, the cells were co-cultured using transwell inserts with a 0.4-μm porous membrane (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) in 24-well or 6-well plates. 3T3-L1 adipocytes (1×10^5 to 1×10^6 cells) were incubated in the lower wells, and RAW264 cells (1×10^4 to 1×10^5 cells) were incubated in the upper wells. After 24 h of co-culture with RAW264 cells, total RNA or total protein was extracted from the 3T3-L1 cells, as described below, and the expression of the indicated genes and proteins was

determined. After 24 h of co-culture with RAW264 cells, 3T3-L1 adipocytes were starved in FBS-free DMEM for 6 h followed by the addition of 10–100 nM mouse GIP (1-42) for 1 or 3 h. The cells were then collected for gene expression analysis.

Hypoxia treatment of 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were incubated in an atmosphere containing ≤2% oxygen for 24 h by using a BIONIX hypoxia incubation kit (Sugiyama-gen, Co. Ltd., Tokyo, Japan). The oxygen concentration was monitored with an oxygen meter (OXY-1, attachment for the kit). The cells were cultured in an atmosphere containing 21% oxygen for 3 or 6 h for reoxygenation. Cobalt chloride (Wako) and deferoxamine mesylate salt (Sigma-Aldrich) (50–200 μM), activators of HIF-1α, were used as hypoxia mimic agents, and cells were collected after a 24-h incubation.

HIF-1α knockdown in adipocytes by RNA interference

Differentiated 3T3-L1 adipocytes were detached from the plates and were transfected with 10 nM of a HIF-1α small interfering RNA (GS15251 GeneSolution siRNA, QIAGEN) or negative control siRNA (4390843, Life Technologies) by using Lipofectamine RNAiMAX (Life Technologies). Optimal transfection conditions were

identified in preliminary experiments, in which the knockdown efficiency at 24 h after transfection was determined.

The siRNA-treated adipocytes were co-cultured with RAW264 cells or incubated in an atmosphere containing ≤2% oxygen for 24 h. After 24 h of co-culture with RAW264 cells, the adipocytes were stimulated with GIP (1-42) for 1 h.

Western blotting

3T3-L1 adipocytes were lysed with CelLytic M (Sigma-Aldrich). Protein concentrations were measured with a BCA Protein Assay (Thermo Fisher Scientific K.K., Yokohama, Japan). Each sample (10–20 μg) was loaded onto a 4%–15% Criterion gel for electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were then transferred to polyvinylidene fluoride membranes and incubated with primary antibodies, followed by an HRP-linked anti-rabbit or anti-mouse IgG secondary antibody (Cell Signaling Technology Japan K.K., Tokyo, Japan). Signals were detected by using an ECL Prime Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan) on a ChemiDoc XRS System (Bio-Rad). Primary antibodies against HIF-1α (NB100-449) and and β-Actin (#4967) were purchased from Novus Biologicals (Littleton, CO) and Cell Signaling Technology Japan K.K individually.

Cytokine analysis by ELISA

3T3-L1 adipocytes were starved in FBS-free DMEM for 6 h and stimulated with GIP (AnaSpec Inc.) for 3 h. Media collected from adipocytes were used for the ELISA assay. Mouse interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) ELISA kits were purchased from Biolegend Inc. (San Diego, CA).

Statistical analysis

Data are expressed as means ± SEM. Student's *t* test was used for single comparisons. One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons. Two-way ANOVA (repeated measures) was used for the ITT analysis. Associations between two parameters were analyzed using Pearson's correlation coefficient. The threshold for significance was $P < 0.05$. All statistical analyses for this study were performed in Prism 8 (GraphPad Software Inc.)

Results

GIP treatment increases proinflammatory gene expression and macrophage infiltration in abdominal adipose tissue in obese (db/db) mice.

In a preliminary experiment, intraperitoneal GIP administration significantly increased blood GIP levels in both misty and db/db mice without affecting blood glucose or insulin levels (Supplementary Fig. 1). Both misty and db/db mice treated with GIP for 1 wk had significantly higher levels of MCP-1 mRNA in the retroperitoneal fat than the corresponding control mice (Fig. 9A). GIP-treated db/db mice, but not misty mice, had significantly more MCP-1 mRNA in the perirenal fat (Fig. 9B) and IL-6 mRNA in the epididymal fat (Fig. 9C) than did the control mice. F4/80 mRNA was significantly higher in GIP-administered misty mice than in the control misty mice, whereas F4/80 expression was similar between the GIP-treated and control db/db groups (Fig. 9D). However, immunohistochemistry showed that GIP-treated db/db mice had more F4/80-positive cells with a "crown-like-structure" (26) in the retroperitoneal fat than did the control db/db mice (Fig. 9E), whereas no notable change was found in the misty mice.

GIP treatment increases blood glucose levels in obese (db/db) mice.

Db/db mice treated with GIP for 3 wk had significantly higher blood glucose levels during

the ITT than did saline-treated control mice (Fig. 10A). After 4 wk of GIP treatment, fasting blood glucose levels were significantly higher in GIP-treated db/db mice than in the control db/db mice (Fig. 10C). Fasting blood insulin levels were similar between the db/db groups (Fig. 10D). Blood glucose levels during ITT (Fig. 10B) and in the fasting condition (Fig. 10C) were similar between the GIP-treated and control misty mice.

GIPR gene expression is correlated with HIF-1α gene expression in adipose tissue in mice.

GIPR and HIF-1α gene expression were significantly higher in db/db mice than in misty mice. GIP treatment for 1 wk significantly increased GIPR (Fig. 11A) and HIF-1α (Fig. 11B) mRNA levels in misty mice, but not in db/db mice . The GIPR mRNA level was positively correlated with the HIF-1α mRNA level in adipose tissue (Fig. 11C), but not in pancreas (Fig. 11D) when mice from all groups (i.e., both strains of mice with and without GIP treatment) were analyzed together.

Gene expression of GIPR, HIF-1α, and VEGFa increases during adipocyte differentiation.

During the differentiation of 3T3-L1 adipocytes, GIPR gene expression slightly increased

at 2 h, declined to baseline again by 24 h, and then dramatically increased and peaked on day 6 (Fig. 12A). CCAAT/enhancer binding protein (CEBP) δ gene expression peaked at 0.5 h and then declined approximately to baseline by 2 h (Fig. 12B). Gene expression of other adipocyte differentiation markers, such as adipocyte protein 2 (aP2) (Fig. 12C), CEBPα (Fig. 12D), peroxisome proliferator-activated receptor (PPAR) γ (Fig. 12E), and sterol regulatory element binding protein (SREBP)-1c (Fig. 12F), peaked 6–8 d after the initiation of differentiation. Like GIPR, HIF-1α and vascular endothelial growth factor a (VEGFa) mRNA also had 2 temporal peaks, at 2 h and 8 d after the initiation of differentiation (Fig. 12G, H).

GIP treatment of mature adipocytes increases proinflammatory adipokine production.

In a preliminary experiment, GIP treatment did not cause cytotoxicity in adipocytes. Treatment of differentiated 3T3-L1 adipocytes on day 6 with GIP significantly increased IL-6 mRNA expression in a dose-dependent manner (Fig. 13A). Treatment of the cells with 100 nM GIP significantly increased the MCP-1 mRNA level (Fig. 13B) and the protein levels of IL-6 and MCP-1 in culture media (Fig. 13C and 13D, respectively).

Co-culture of adipocytes with macrophages, but not with macrophage-conditioned medium, increases GIPR gene expression.

Co-culture of 3T3-L1 adipocytes with RAW264 macrophages substantially upregulated GIPR mRNA levels in the adipocytes (Figs. 14A and 14B). Neither conditioned medium from RAW264 cells nor proinflammatory cytokines (IL-6, TNFα, and plasminogen activator inhibitor [PAI] 1) affected GIPR gene expression (Figs. 14A and 14B), although the co-culture significantly increased expression of proinflammatory genes (MCP-1, IL-6, and PAI1) and significantly decreased leptin and adiponectin gene expression in the adipocytes (Fig. 14C). Only culture with IL-1β significantly increased GIPR mRNA expression in the adipocytes (Fig. 14B).

GIP treatment of adipocytes co-cultured with macrophages aggravates adipokine dysregulation.

In 3T3-L1 adipocytes cultured with RAW264 macrophages, treatment with GIP (100 nM) significantly increased mRNA levels of IL-6 (Fig. 15A) and PAI1 (Fig. 15B) and significantly decreased those of leptin (Fig. 15C) and adiponectin (Fig. 15D).

Hypoxia and HIF-1α activators increase GIPR mRNA expression in adipocytes.

Exposure to hypoxia (2% O_2) significantly augmented GIPR gene expression in 3T3-L1 adipocytes; this increase was diminished by reoxygenation in a time-dependent manner (Fig. 16A). Hypoxia significantly increased the mRNA levels of glucose transporter 1 (GLUT1) (Fig. 16B), but did not affect the levels of HIF-1α mRNA (Fig. 16C). Both $CoCl_2$ and deferoxamine, which activate HIF-1α (7, 13), significantly increased gene expression of GIPR (Fig. 16D) and GLUT1 (Fig. 16E) in a dose-dependent manner.

HIF-1α knockdown diminishes both macrophage- and hypoxia-induced GIPR gene expression in adipocytes.

Culture of 3T3-L1 adipocytes with RAW264 macrophages (Fig. 17A) or under hypoxic conditions (Fig. 17B) increased HIF-1α protein levels in the adipocytes. Downregulation of HIF-1α mRNA in the adipocytes by a specific siRNA (Fig. 17C) significantly diminished the increase in GIPR gene expression due to either co-culture with macrophages (Fig. 17D) or hypoxia (Fig. 17E) as well as the increase in GLUT1 gene expression due to hypoxia (Fig. 17F).

HIF-1α knockdown diminishes the GIP-induced gene expression of IL-6 in adipocytes co-cultured with macrophages.

Downregulation of HIF-1α mRNA by specific siRNA (Fig. 18A) decreased GIPR gene expression in the control and in GIP-treated adipocytes co-cultured with RAW264 macrophages (Fig. 18B). HIF-1α gene silencing diminished the increase in IL-6 mRNA expression induced by GIP treatment in the adipocytes (Fig. 18C).

Discussion

This study had three major findings. First, chronic GIP treatment induced adipose tissue inflammation, as characterized by increased gene expression of MCP-1 and IL-6 and macrophage infiltration into the tissue, and thereby impaired insulin sensitivity in obese mice. Second, mature adipocytes cultured either with macrophages or under hypoxic conditions had increased GIPR and proinflammatory gene expression, and GIP treatment of the adipocytes further increased proinflammatory gene expression. Finally, HIF-1α was involved in the macrophage-induced expression of the GIPR gene and proinflammatory genes in adipocytes. These findings support the hypothesis that increased GIP signaling plays a significant role in adipose tissue inflammation and insulin resistance in obese mice, and that the proinflammatory GIP signaling is mediated by a HIF-1α–dependent pathway that augments GIPR and proinflammatory gene expression in adipocytes.

This is the first study to show evidence that chronic elevation of blood GIP concentrations stimulates proinflammatory gene expression and macrophage infiltration into adipose tissue and impairs insulin sensitivity in mice. These results in mice support the view of Nie et al. [23] and Timper et al. [24], who showed that GIP impairs insulin signaling by inducing adipocyte inflammation in cultured adipocytes. In contrast to the

significant increase in MCP-1 and IL-6 gene expression caused by intraperitoneal GIP treatment, neither TNFα nor IL-1β gene expression were changed in my study. Consistent with this, GIP enhanced TNFα gene expression in GIPR-overexpressing adipocytes [23] and that of IL-1β in human adipocytes [24] less than quarter as much and half as much, respectively, as it enhanced IL-6 gene expression. Therefore, the effect of increased GIP signaling on TNFα or IL-1β gene expression may not be meaningful *in vivo*.

The increase of fasting blood glucose without an accompanying change in insulin levels in GIP-treated obese mice suggests that GIP treatment may affect hepatic glucose production. In fact, expression levels of gluconeogenesis-related genes in the liver, such as fructose-1, 6-bisphosphatase 1 (FBP1) and phosphoenolpyruvate carboxykinase (PEPCK), were significantly increased in the GIP-treated obese mice (Supplementary Fig. 2). Therefore, hepatic insulin sensitivity was also impaired in db/db mice by treatment with GIP. Because no functional GIPR has been found in liver so far, the results observed in liver seem to be indirect effects of GIP.

In contrast to its effects in obese (db/db) mice, GIP treatment did not increase MCP-1 gene expression in the perirenal fat or that of IL-6 in the epididymal fat in lean (misty) mice, and did not significantly ($P = 0.066$) impair their insulin sensitivity. Even though GIP stimulated MCP-1 and F4/80 gene expression in the retroperitoneal fat of the

lean mice, gene expression of proinflammatory biomarkers (MCP-1, IL-6, and F4/80) and adipose infiltration of macrophages into the adipose tissues were still much less in lean mice than in obese mice. These results indicate that the adipose tissue inflammation induced by increased concentrations of GIP in the blood might not be large enough to cause insulin resistance in lean mice. Whereas GIPR and HIF-1α gene expression levels were similar between GIP-treated lean and obese mice, blood glucose, insulin, and GIP levels were significantly higher in obese mice than in lean mice (Supplementary Fig. 1). Thus, increased GIP signaling may act in coordination with increased blood glucose, insulin, or GIP levels in obese mice to stimulate adipose tissue inflammation and cause insulin resistance.

In my *in vitro* study, GIPR gene expression exhibited a biphasic increase during adipocyte differentiation, and peaked on day 6 after the induction of differentiation. GIP stimulated IL-6 and MCP-1 gene expression and dramatically increased the secretion of proinflammatory proteins from the adipocytes when GIPR expression was at its peak in the adipocytes. Nie *et al*. [23] showed that GIP did not increase IL-6 and MCP-1 gene expression in 3T3-L1 adipocytes due to low endogenous expression of GIPR, but did stimulate proinflammatory gene expression in GIPR-overexpressing adipocytes. My results are compatible with their findings and suggest that adequate GIPR expression is

necessary for the proinflammatory GIP signaling in adipocytes.

Little has been known about the regulation of GIPR expression in mature adipocytes, although PPAR-γ has been shown to upregulate GIPR expression during adipocyte differentiation [58]. Of the most interest, my study provided the first evidence that GIPR expression increased dramatically along with adipocyte differentiation and is further enhanced in mature adipocytes exposed to macrophages or hypoxia. This enhancement was mediated by HIF-1α signaling in the cells. GIPR expression synchronized not only with differentiation markers such as aP2, CEBPα, PPARγ, and SREBP-1c, but also with VEGFa expression in adipocytes, which suggests that increased HIF-1α signaling may promote GIPR expression along with adipocyte differentiation.

HIF-1α signaling is activated under hypoxic conditions and induces genes related to angiogenesis, erythropoiesis, and glycolysis [49]. Hypoxia has been shown to impair insulin sensitivity in adipocytes [52]. Adipose tissue–specific deletion of HIF-1α protects mice from adipocyte inflammation and insulin resistance induced by a high-fat diet [53, 55]. In the present study, GIPR gene expression in adipose tissue was positively correlated with HIF-1α gene expression. In addition, HIF-1α knockdown in adipocytes inhibited GIPR gene expression. These results together suggest that the HIF-1α–mediated increase in GIPR expression may play a major role in adipocyte inflammation and insulin

resistance.

Stimulation of HIF-1α signaling in adipocytes does not seem to involve humoral factors from macrophages, because neither the conditioned medium nor the proinflammatory cytokines derived from macrophages increased GIPR expression in the cells. Nalwaya *et al.* [59] showed that oxygen consumption is dramatically increased in activated macrophages *in vitro*. In addition, contact with macrophages has been shown to dramatically increase fatty acid release from adipocytes [57], which uncouples adipocyte respiration and increased oxygen consumption [55]. The crosstalk between adipocytes and macrophages has been suggested to increase O_2 consumption in both cell types [55, 59]. Therefore, I consider that adjacent macrophages play an important role in the progression of adipose tissue hypoxia. However, further study is needed to clarify the mechanism for the induction of adipose tissue hypoxia by macrophages.

The results of the present study suggest that a vicious cycle involving GIP signaling and macrophage infiltration aggravates adipose tissue inflammation as follows: 1) Expression of GIPR is increased along with adipocyte differentiation, which leads to increased GIP signaling in adipose tissue; 2) Increased GIP signaling in adipocytes induces proinflammatory gene expression, which leads to macrophage infiltration in adipose tissue; 3) Macrophage infiltration stimulates HIF-1α signaling in adipose tissue,

which leads to a further increase in GIPR expression. This vicious cycle seems to be extremely important for understanding the pathophysiology of insulin resistance in obesity. Proinflammatory GIP signaling may be further exacerbated during the progression of obesity and adipose tissue inflammation. Downregulation of HIF-1α might be a beneficial strategy for interrupting the vicious cycle of proinflammatory GIP signaling in inflamed adipocytes.

The present study had some limitations. Whereas I found that HIF-1α signaling regulated GIP signaling in cultured adipocytes, I have not yet been able to provide evidence on its role in proinflammatory GIP signaling within adipose tissues. Although GIPR and HIF-1α gene expression were positively correlated in adipose tissue, GIP administration increased adipose GIPR and HIF-1α gene expression only in lean mice, and not in obese mice. Conversely, macrophage infiltration in adipose tissue and impairment of insulin sensitivity were observed in obese mice, but not in lean mice. Considering that obese mice have significantly higher basal GIPR and HIF-1α expression in adipose tissue than lean mice, transcriptional upregulation of HIF-1α may be saturated in the obese adipose tissue, which results in a lack of further induction of HIF-1α gene expression by GIP. Examination of either HIF-1α protein stabilization or gene expression of biomarkers of HIF-1α signaling will be needed to clarify whether increased blood GIP

stimulates HIF-1α signaling in obese adipose tissues.

Further studies are also needed to clarify the role of HIF-1α in GIP-mediated adipocyte dysregulation, since I relied on the effects of HIF-1α silencing in adipocytes, in particular the reduction of GIP-induced IL-6 expression, to understand the function of HIF-1α (Fig. 10C). Because GIPR expression was dramatically decreased in HIF-1α–silenced adipocytes cultured with macrophages, the GIP-mediated decrease in adiponectin should be prevented in HIF-1α–depleted adipocytes. However, neither MCP-1 nor adiponectin expression was changed in the same experimental conditions shown in Fig. 10C.

Naitoh *et al.* [56] showed that a high-fat diet increased adiponectin expression in GIPR-deficient mice, but decreased adiponectin in wild-type mice, compared with those on a control diet. The diet dramatically increased visceral fat accumulation in wild-type mice, but not in GIPR-deficient mice. Because visceral fat accumulation increases HIF-1α expression in the adipose tissue, HIF-1α might be depleted in the adipose tissue of GIPR-deficient mice and of mice fed a control diet as compared with wild-type mice fed a high-fat diet. A high-fat diet increases blood GIP compared to a control diet in wild-type mice. Accordingly, I hypothesize that GIP decreases adiponectin expression in adipose tissue, but HIF-1α depletion may prevent the GIP-mediated decrease in

adiponectin expression. Further investigations regarding HIF-1α–mediated proinflammatory GIP signaling in obese adipose tissues, including experiments to determine whether GIP-induced adipose tissue inflammation would be diminished in mice depleted of adipose HIF-1α, will promote the understanding of the pathophysiology of adipose tissue inflammation and insulin resistance induced by obesity or a high-fat diet.

In conclusion, this study provides evidence that increased blood levels of GIP stimulate adipose tissue inflammation *in vivo* and that HIF-1α plays an important role in regulating proinflammatory GIP signaling in adipocytes. Because the blood GIP level is elevated in obesity [19, 21] and after ingestion of a high-fat diet [14, 15], a decrease in GIP signaling in obesity or in the postprandial state may be a beneficial strategy for reducing adipose tissue inflammation and improving insulin sensitivity. My results also disclose a novel factor, HIF-1α, as an underlying contributor to GIP-induced adipose inflammation via its upregulation of GIPR. This makes HIF-1α a therapeutic target for the development of GIP-based treatments for obesity and type 2 diabetes. Further studies are needed to clarify the molecular mechanism of HIF-1α–induced GIPR expression in adipocytes and the efficacy of regulating GIP or HIF-1α signaling in adipose tissues for improvement of glucose homeostasis.

Table and Figures

Table 1. Composition of the experimental diet

Ingredients	(%)
Potato starch	66.5
Triglycerides*	5
Casein	20
Cellulose	4
Mineral mixture	3.5
Vitamin mixture	1
Energy (kcal/g)	4.0

* The fatty acid composition of the triglycerides was 35.24% oleic acid, 2.00% stearic acid, 48.15% linoleic acid, and 14.61% others.

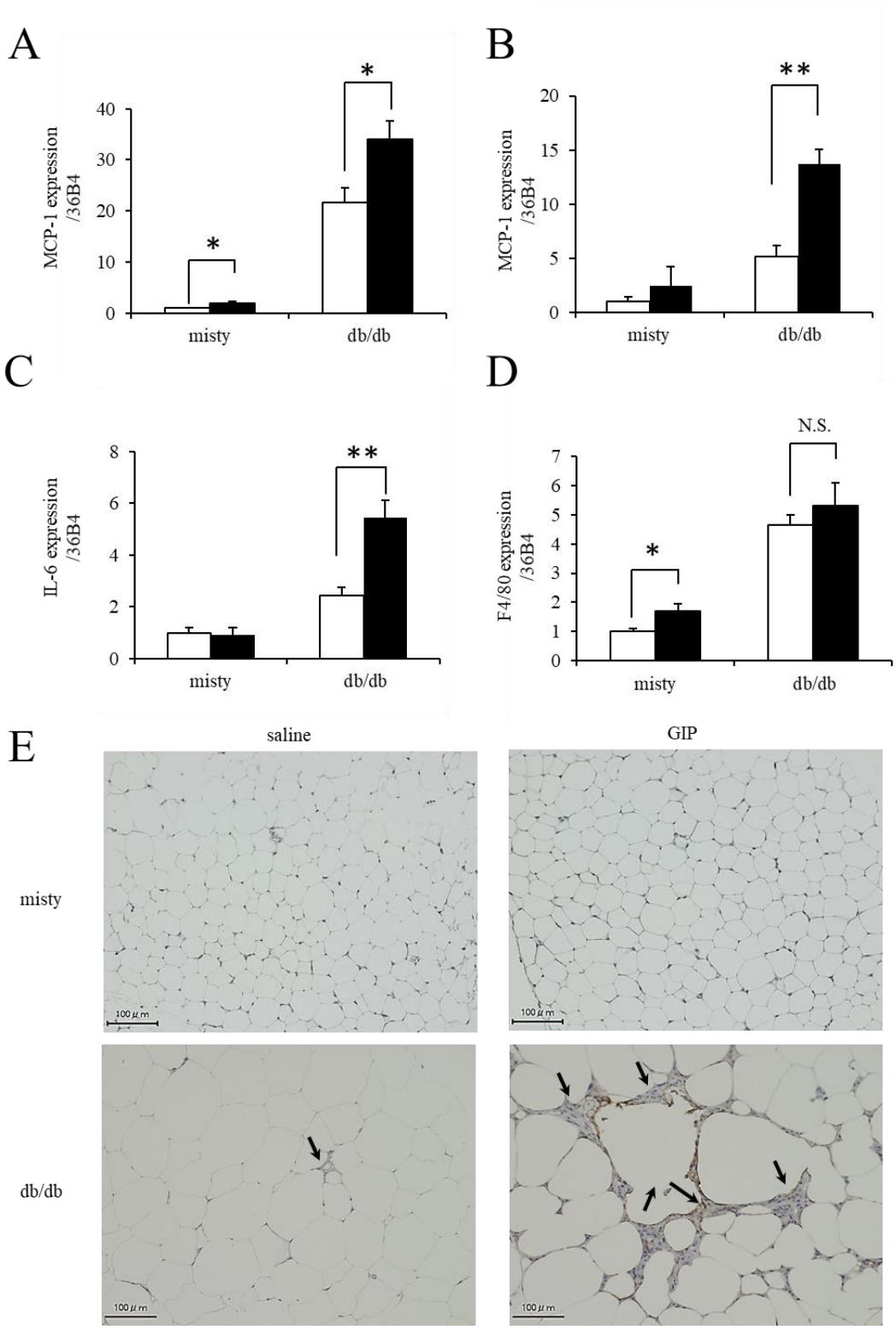


Fig. 9 GIP treatment induces adipose tissue inflammation in mice. (A–D) GIP (10 nmol/kg body weight) was administered intraperitoneally to lean (misty) and obese

(db/db) mice twice daily for 1 wk. Gene expression levels of MCP-1 in (A) retroperitoneal and (B) perirenal fat, (C) IL-6 in epididymal fat, and (D) F4/80 in retroperitoneal fat were determined with qRT-PCR. Open bars = saline group; closed bars = GIP group. Data are presented as means ± SEM, n = 6. * $P < 0.05$, ** $P < 0.01$; N.S., not significant (Student's *t* test). (E) F4/80 protein localization in retroperitoneal fat was examined with immunohistochemistry in misty and db/db mice administered either saline or GIP. Arrows indicate "crown-like-structures".

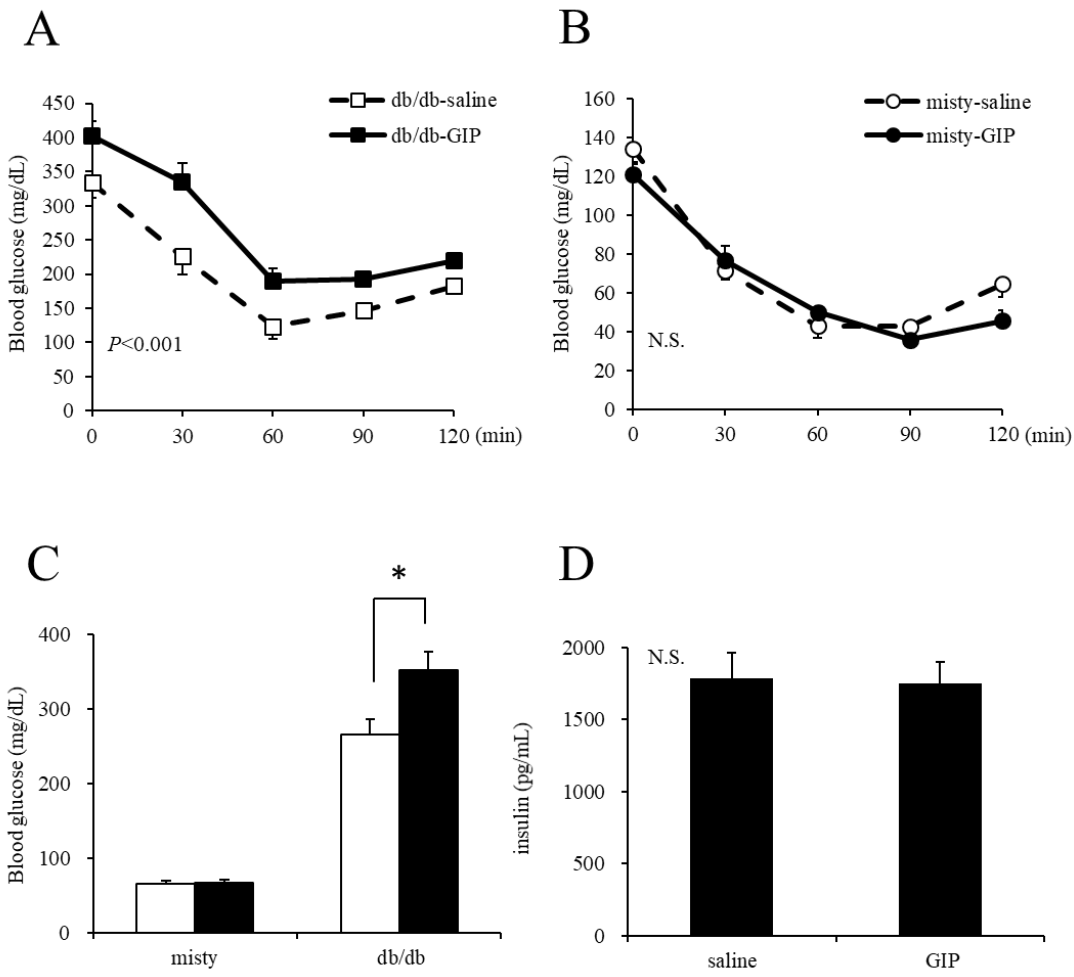


Fig. 10 GIP treatment increases blood glucose in mice. (A, B) GIP (5 nmol/kg body weight) or saline was administered intraperitoneally to db/db and misty mice daily for 3 wk. The time course of blood glucose changes before (time 0) and for 2 h after insulin administration in the insulin tolerance test (ITT) is shown for (A) db/db and (B) misty mice. Data are presented as means ± SEM, n = 6, and were analyzed by repeated measures two-way ANOVA. N.S., not significant. (C) Fasting blood glucose levels and (D) insulin levels were measured after 4 wk of GIP administration. Open bars = saline group; closed bars = GIP group. Data are presented as means ± SEM, n = 6. * $P < 0.05$ (Student's *t* test).

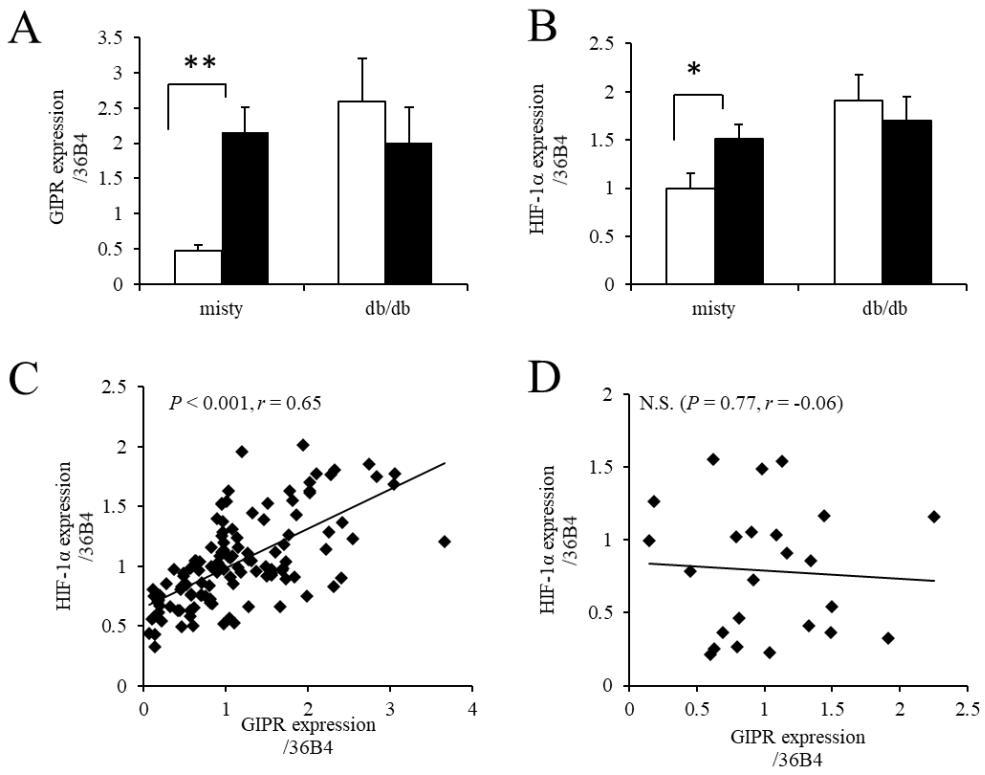


Fig. 11 GIPR and HIF-1α gene expression are correlated in adipose tissue but not in pancreas in mice. Adipose tissues (epididymal, mesenteric, perirenal, retroperitoneal, and inguinal) and pancreas were removed from misty and db/db mice after 1 wk of saline or GIP administration. GIPR (A) and HIF-1α (B) in mesenteric fat tissues were examined in misty and db/db mice. Open bars = saline group; closed bars = GIP group. Data are presented as means ± SEM, n = 6. * $P < 0.05$, ** $P < 0.01$ (Student's *t* test). (C, D) Gene expression levels of GIPR and HIF1α in (C) the adipose tissues (n = 120) and (D) pancreas (n = 24) of both misty and db/db mice were determined with qRT-PCR. A Pearson's correlation coefficient (*r*) was obtained to estimate the linear correlation between GIPR and HIF1α expression. N.S., not significant.

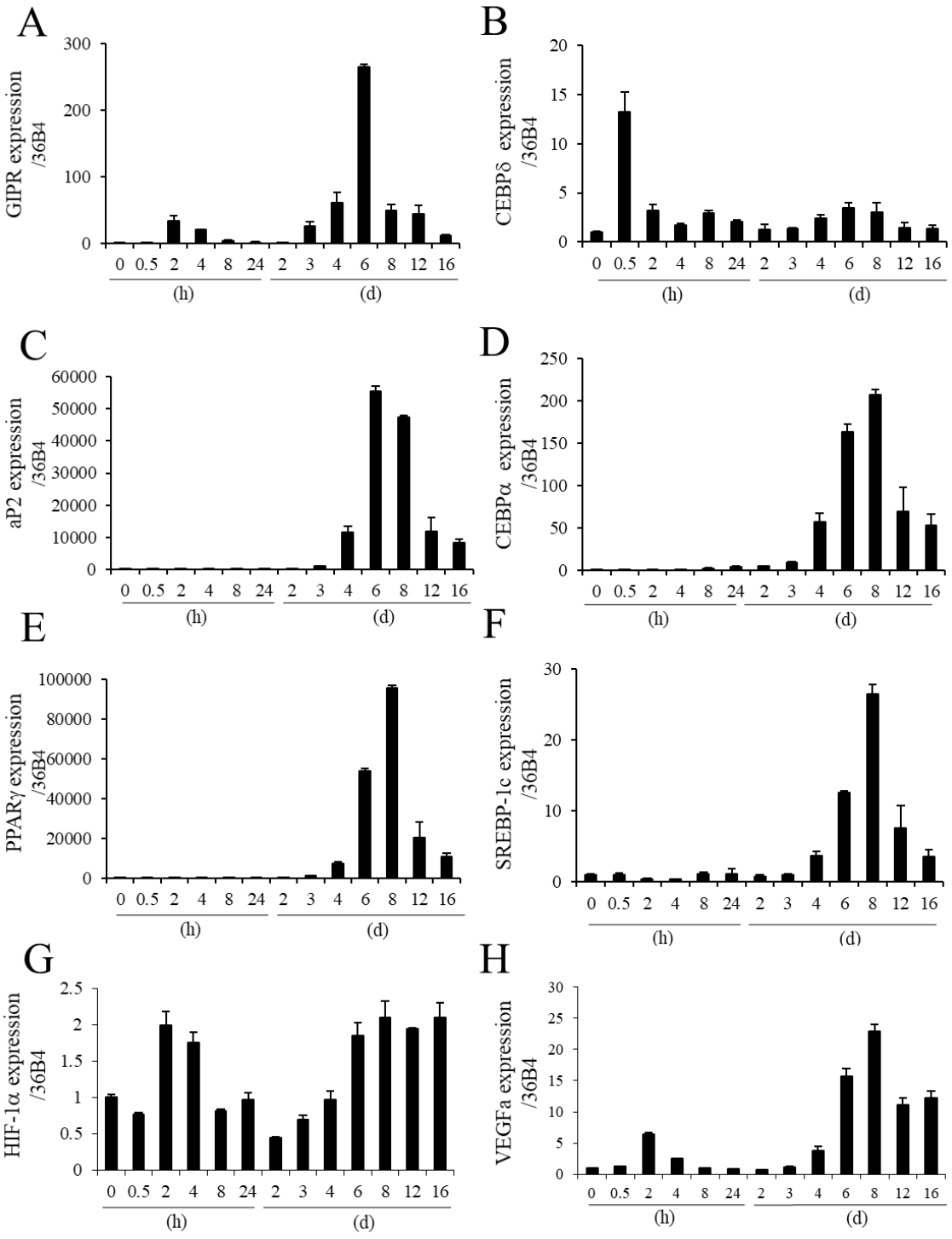


Fig. 12 Time course of gene expression during adipocyte differentiation. Premature 3T3-L1 adipocytes were differentiated with 10 mg/L insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methly-xanthine for 2 d, followed by 5 mg/L insulin in

DMEM for another 2 d, and with DMEM without insulin for a further 12 d. Gene expression levels for (A) GIPR, (B) C/EBPδ, (C) aP2, (D) C/EBPα, (E) PPARγ, (F) SREBP1c, (G) HIF-1α, and (H) VEGFa at the indicated time points were examined with qRT-PCR. Data are presented as means ± SEM, n = 3.

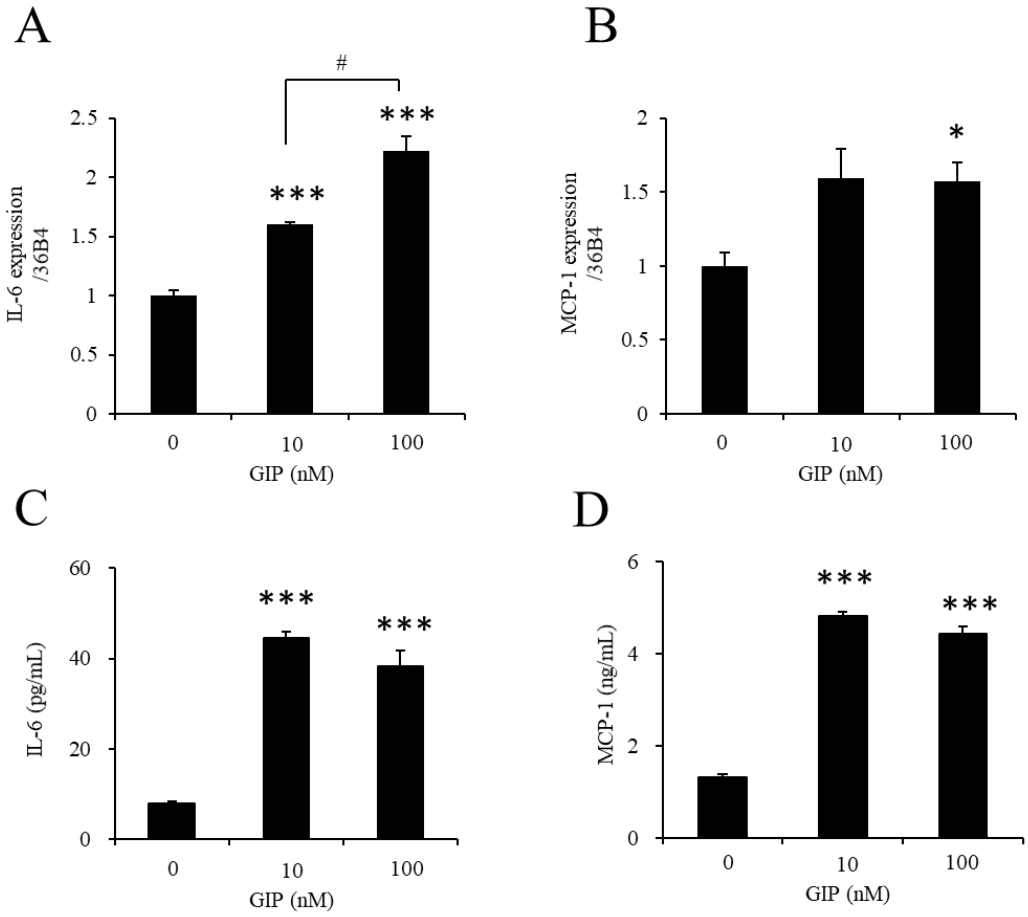


Fig. 13 Proinflammatory cytokine expression in differentiated adipocytes in response to GIP. Differentiated 3T3-L1 adipocytes (on day 6) were cultured in FBS-free DMEM for 6 h followed by treatment with 0, 10, or 100 nM GIP. After 1 h or 3 h of GIP treatment, gene expression levels for (A) IL-6 (after 1 h) and (B) MCP-1 (after 3 h) were determined with qRT-PCR. (C, D) After 3 h, the culture media were collected, and the concentrations of (C) IL-6 and (D) MCP-1 were measured. Data are presented as means ± SEM, n = 4. * $P < 0.05$, *** $P < 0.001$ vs. Control (GIP 0 nM); # $P < 0.05$ between the indicated groups (ANOVA with Tukey's multiple comparison test).

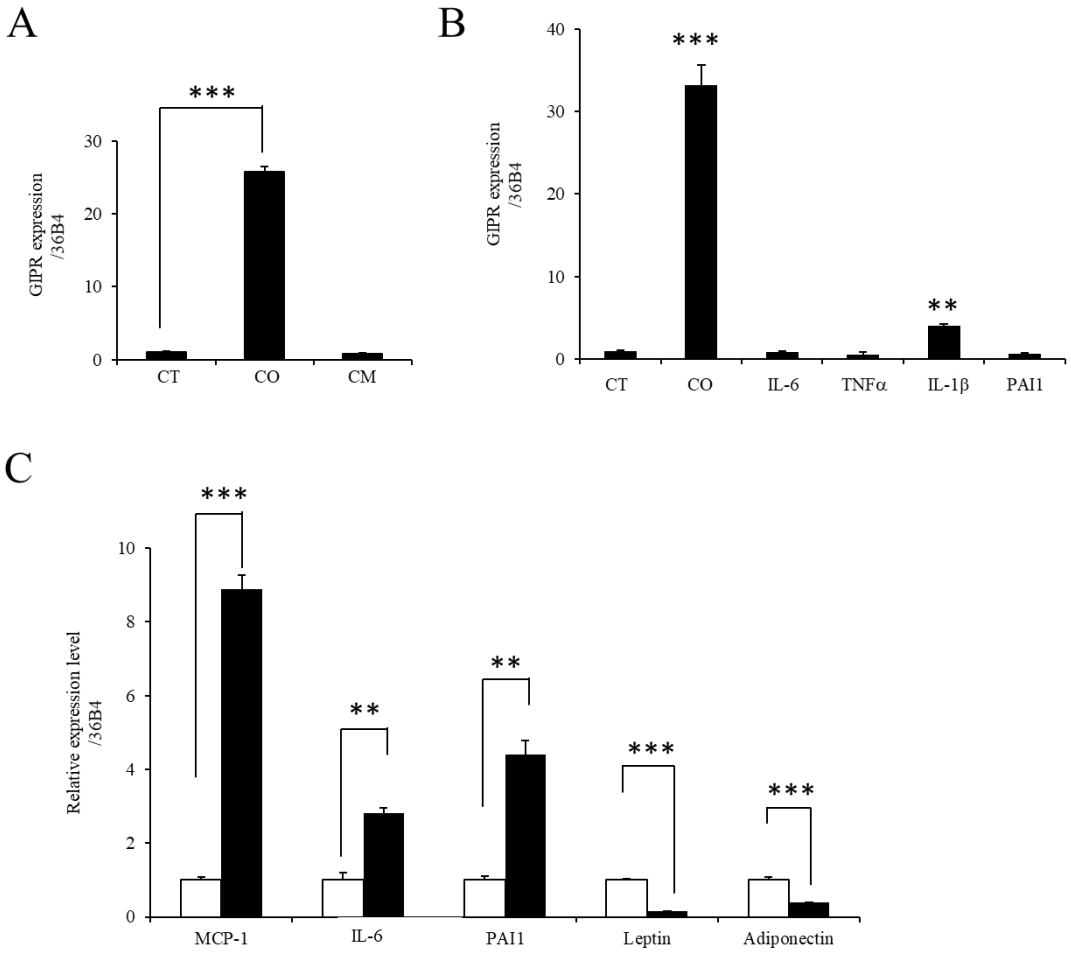


Fig. 14 Gene expression in adipocytes cultured with or without macrophages or macrophage-conditioned media. (A) Differentiated 3T3-L1 adipocytes were cultured with (CO) or without (CT) RAW264 macrophages (1×10^5 cells/well), or with conditioned media (CM) from RAW264 macrophages. Gene expression of GIPR was determined with qRT-PCR after 24 h of incubation. (B) 3T3-L1 adipocytes were cultured with (CO) or without (CT) RAW264 macrophages (1×10^5 cells/well) or treated with IL-6 (5 ng/mL), TNFα (10 ng/mL), IL-1β (1 ng/mL), or PAI1 (10 μg/mL) for 24 h. (C) Differentiated 3T3-L1 adipocytes were cultured with or without RAW264 macrophages

(1×10^5 cells/well), and gene expression of MCP-1, IL-6, PAI1, leptin, and adiponectin were determined with qRT-PCR after 24 h of incubation. Open bars = without RAW264; closed bars = with RAW264. Data are presented as means ± SEM, n = 4. ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test).

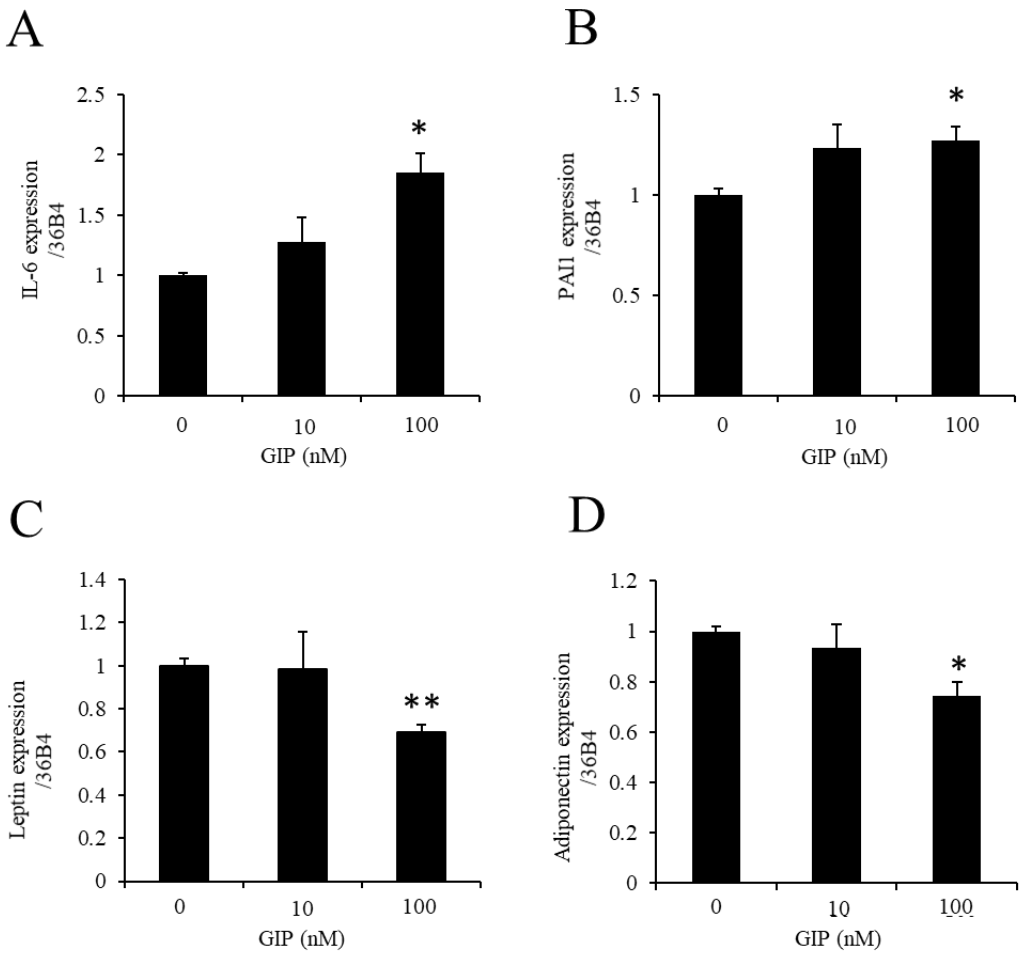


Fig. 15 GIP induces adipocyte dysregulation in macrophage coculture. Differentiated 3T3-L1 adipocytes were cultured with RAW264 macrophages (1×10^5 cells/well) for 24 h and stimulated with GIP (0, 10, or 100 nM) for 1 or 3 h. Gene expression of IL-6 (after 1 h; A) and PAI1 (B), leptin (C), and adiponectin (D) (after 3 h) were determined with qRT-PCR. Data are presented as means ± SEM, n = 4. * $P < 0.05$, ** $P < 0.01$ vs. GIP 0 nM (ANOVA with Tukey's multiple comparison test).

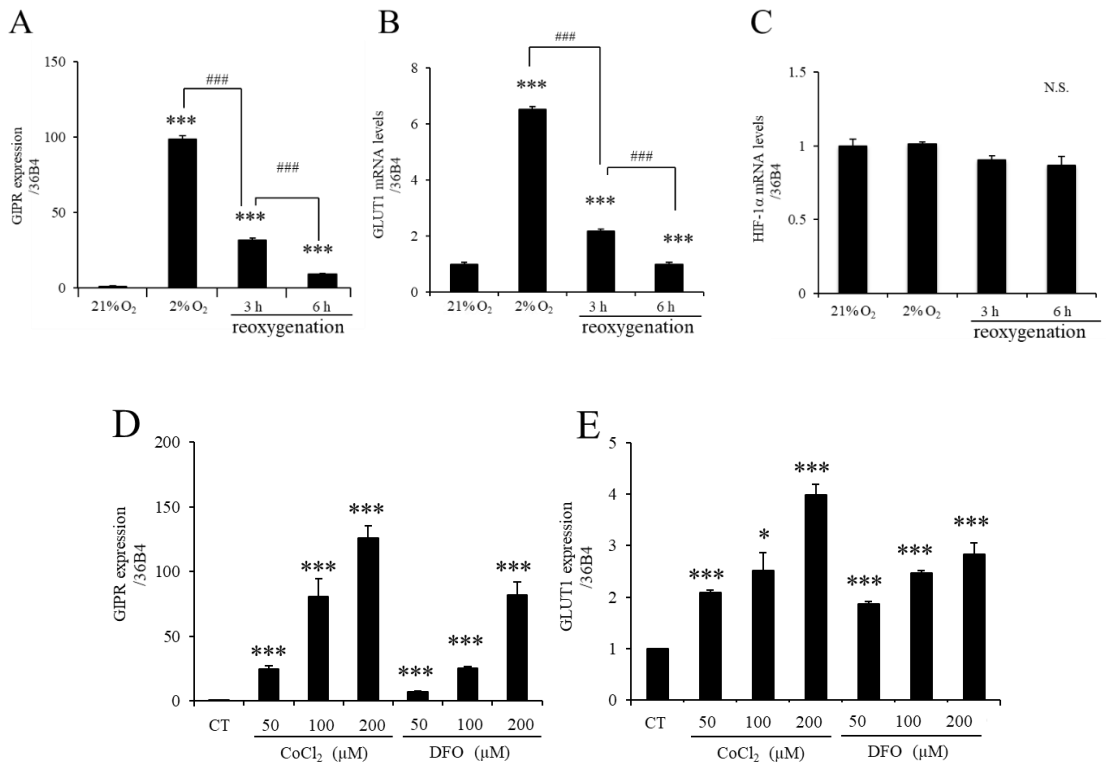


Fig. 16 Hypoxia and HIF-1α activators increase GIPR gene expression in adipocytes.

(A–C) 3T3-L1 adipocytes were cultured in either 21% or 2% O_2, or 2% O_2 followed by 21% O_2 for 3 or 6 h (reoxygenation). (D, E) 3T3-L1 adipocytes were incubated for 24 h with $CoCl_2$ or deferoxamine (DFO) at 50, 100, or 200 μM. Gene expression of GIPR (A, D), GLUT1 (B, E), and HIF-1α (C) was determined with qRT-PCR. Data are presented as means ± SEM, n = 4. * $P < 0.05$, *** $P < 0.001$ vs. 21% O_2 (A, B) or control (CT; D, E); ### $P < 0.001$ between the indicated groups (ANOVA with Tukey's multiple comparison test).

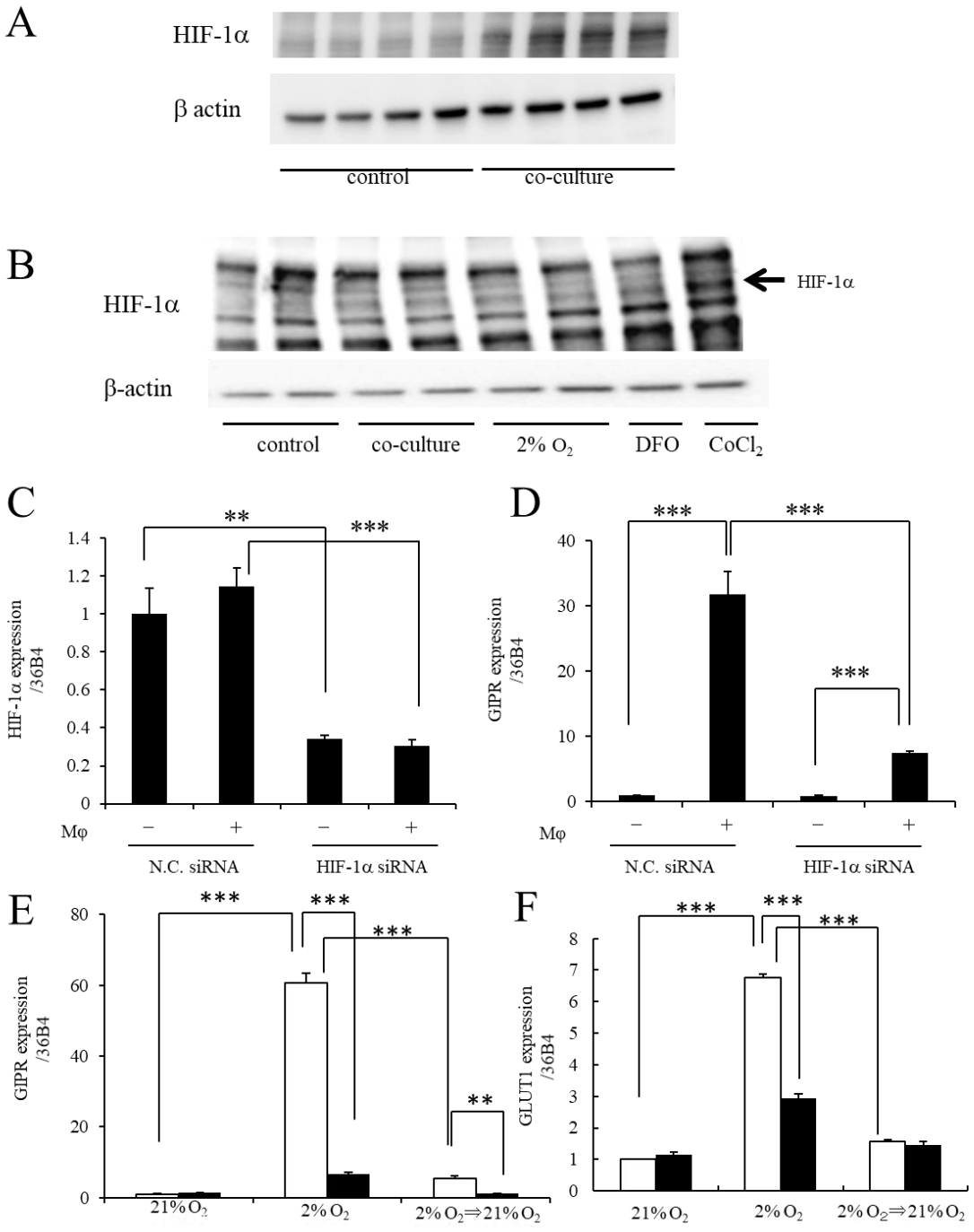


Fig. 17 Knockdown of HIF-1α downregulates GIPR gene expression in adipocytes cultured with macrophages. (A, B) 3T3-L1 adipocytes were cultured (A, B) with RAW264 macrophages (1×10^5 cells/well), or (B) under 2% O_2, or with $CoCl_2$ or DFO

200 μM for 24 h. The HIF-1α protein level was analyzed by SDS-PAGE and Western blot. (C–E) An HIF-1α siRNA or a negative control (N.C.) siRNA was introduced into 3T3-L1 adipocytes and incubated for 24 h. The cells were cultured (C, D) with RAW264 macrophages (1×10^5 cells/well) or (E, F) under 2% O_2 for another 24 h. (E) Some adipocytes cultured under 2% O_2 were reoxygenated in 21% O_2. Gene expression of (C) HIF-1α, (D, E) GIPR, and (F) GLUT1 was determined with qRT-PCR. (E, F) Open bars = control siRNA; closed bars = HIF-1α siRNA. Data are presented as means ± SEM, n = 4. ** $P < 0.01$, *** $P < 0.001$ (ANOVA with Tukey's multiple comparison test).

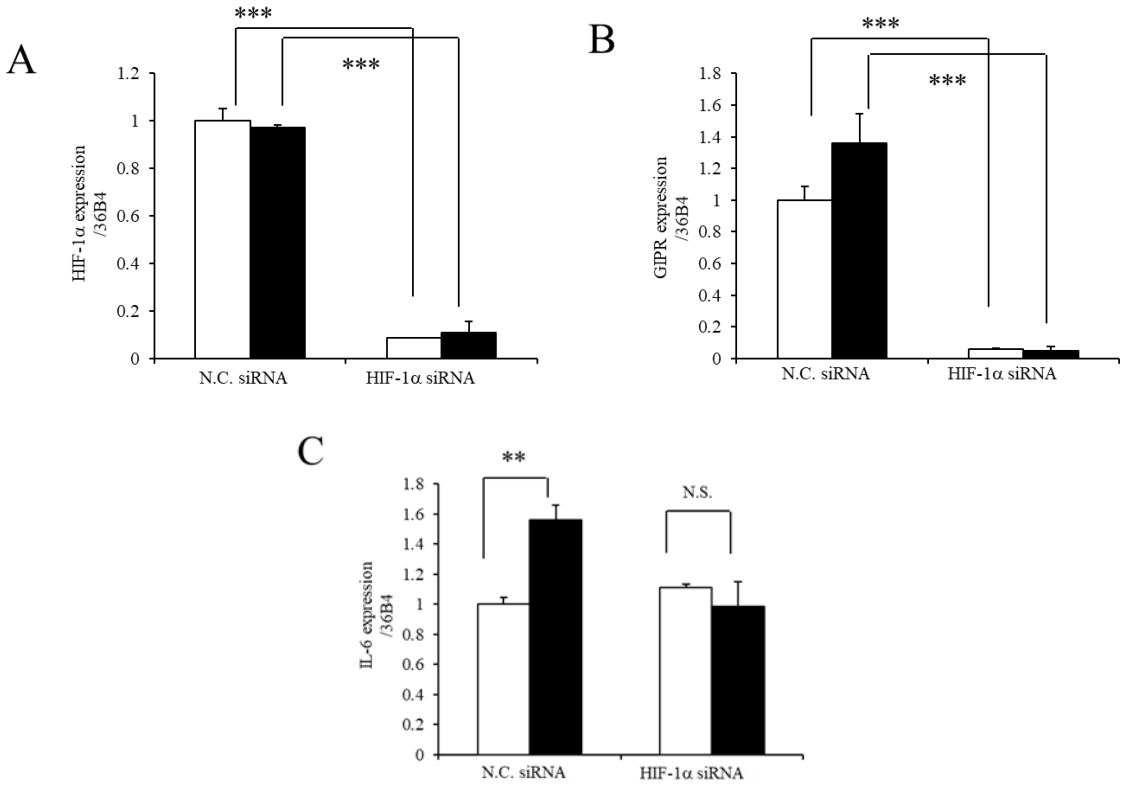
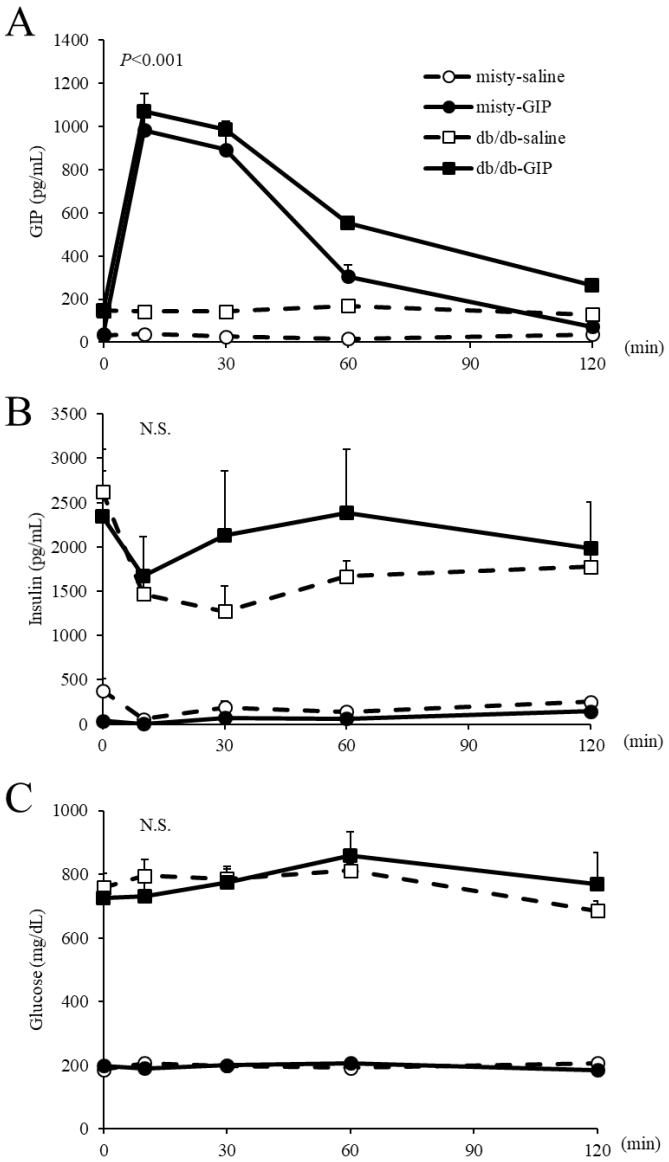


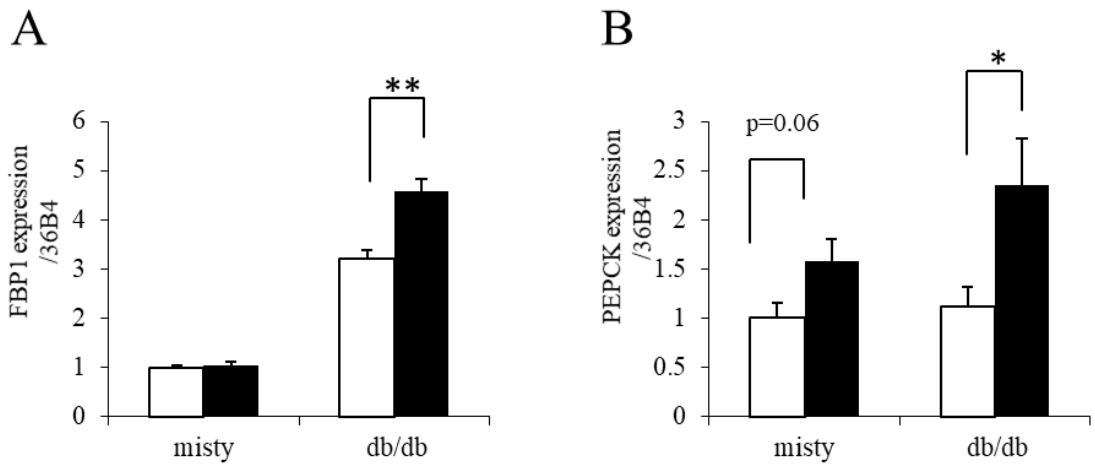
Fig. 18 HIF-1α gene silencing decreases GIPR expression and inhibits GIP-induced IL-6 expression in adipocytes cultured with macrophages. An HIF-1α siRNA or negative control (N.C.) siRNA was introduced into 3T3-L1 adipocytes. and the cells were incubated for 24 h and then cultured with RAW264 macrophages (1×10^5 cells/well) for 24 h and stimulated with 100 nM GIP for 1 h. Gene expression of (A) HIF-1α, (B) GIPR, and (C) IL-6 was determined with qRT-PCR. Open bars = control group; closed bars = GIP-treated group. Data are presented as means ± SEM, n = 4. ** $P < 0.01$, *** $P < 0.001$; N.S., not significant (ANOVA with Tukey's multiple comparison test)



Supplementary Fig. 1 Time course of blood GIP, insulin, and glucose after GIP administration.

GIP (10 nmol/kg body weight) was administered intraperitoneally to obese (db/db) and lean (misty) mice twice daily for 1 wk. Plasma GIP (A), insulin (B), and glucose (C) were measured before (0) and 10, 30, 60, and 120 min after GIP injection. Data are presented as means ± SEM, n = 6, and were analyzed by repeated measures two-way

ANOVA: P < 0.001 between the misty saline and GIP groups and between the db/db saline and GIP groups; N.S., not significant between the misty saline and GIP groups, and between the db/db saline and GIP groups.



Supplementary Fig. 2 Effect of GIP administration on gluconeogenesis-related gene expression in the liver.

GIP (10 nmol/kg body weight) was administered intraperitoneally to lean (misty) and obese (db/db) mice twice daily for 1 wk. Gene expression levels of fructose-1, 6-bisphosphatase 1 (Fbp1; A), and phosphoenolpyruvate carboxykinase (PEPCK; B) in liver were determined with qRT-PCR. Open bars = saline group; closed bars = GIP group. Data are presented as means ± SEM, n = 6. * $P < 0.05$, ** $P < 0.01$, N.S., not significant (Student's *t* test).

General Discussion

Obesity, caused by the imbalance of energy storage and expenditure, can lead to type 2 diabetes and increased risk of chronic diseases such as cardiovascular disease and cancers [1, 2]. In obese state, adipogenesis exceeds lipolysis, and the adipose tissue undergoes dynamic remodeling by increasing number and size of adipocytes. At the pathological stage, rapid enlargement of the adipocytes contributes to the formation of hypoxia in the microenvironment, and M1-stage macrophages infiltrate, which further stimulates adipose tissue inflammation and insulin resistance. Thus, repressing adipose tissue from pathological expansion by stimulating lipolysis and preventing adipose tissue inflammation is critically important for the improvement of obesity and prevention of following health problems.

Understanding the molecular mechanism underlying adipose tissue remodeling may lead to the identification of novel, therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation. To provide new insights of improving obesity and metabolic syndrome, I focused on the pathological expansion of adipose tissue in this study, examined the lipolytic effects of tea catechins with their mechanisms in chapter 1, and revealed the physiological functions of GIP signaling in the pathogenesis of adipose tissue inflammation in chapter 2.

In chapter 1, I revealed that green tea catechins stimulate lipolysis in the presence

of norepinephrine via a PKA-dependent pathway in 3T3-L1 adipocytes. The results suggest that dietary tea catechins, especially when combined with physical activity, might have the potential to stimulate body fat reduction. In chapter 2, I clarified that chronic GIP treatment induces adipose tissue inflammation, as characterized by macrophage infiltration into the adipose tissue in obese mice. The most valuable point of the study is the discovery of a novel factor HIF-1α on the regulation of GIPR expression by either hypoxia or macrophage infiltration, which mediates inflammatory GIP signaling in adipose tissue and impairs insulin resistance in obese mice.

As summarized in Fig. 19, my studies showed that green tea catechins could be useful for reduction of obesity by stimulating lipolysis and prevention of adipose tissue pathological expansion. On the other hand, in the pathological stage, where M1-stage macrophages infiltrates and hypoxia induced, GIP signaling further contributes to adipose tissue inflammation through elevated HIF-1α-induced GIPR expression. These findings shed the light on green tea catechins and GIP on prevention and improvement of obesity. Daily consumption of green tea catechins and intake of meal that decreases GIP secretion could be useful not only for prevention of body fat accumulation but also for reduction of obesity.

The limitation of the present study is that the experiments are mainly conducted

in vitro, which need to be examined *in vivo*. Recently, adipose tissue-specific GIPR knockout (GIPR $^{adipo-/-}$) mice has been generated [60], and plasma levels of IL-6 and insulin resistance were reduced in high fat diet fed GIPR $^{adipo-/-}$ mice, which are consistent with my data and indicates the critical role of GIPR signaling in high fat diet induced adipose tissue inflammation and insulin resistance. Interestingly, authors revealed that GIP induced IL-6 mRNA expression and production in adipocytes can be increased in the presence of TNF α [60]. In my study, single TNF α stimulation did not affect GIPR expression, thus the increase of IL-6 induced by TNF α maybe mediated by other signaling which remained to be elucidated.

Finally, I am grateful to be able to disclose these new molecular mechanisms which may provide new insights on therapeutic strategies to prevent or treat obesity and contribute to the progress of biological sciences

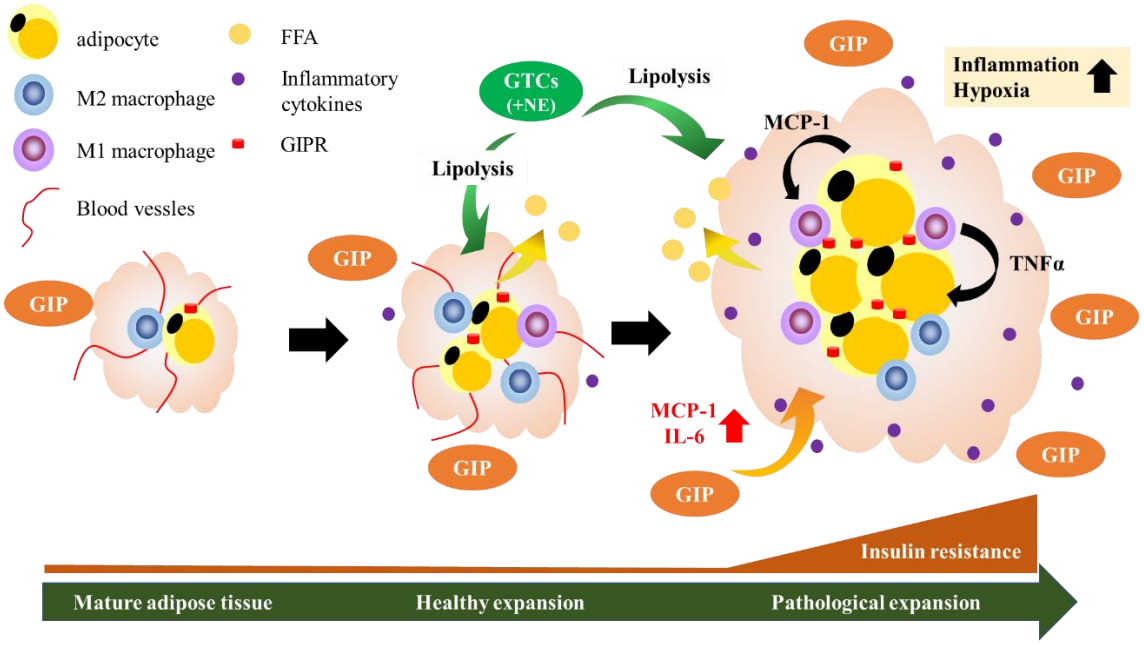


Fig. 19 Summary of this study

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