Original Article

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Guanylyl cyclase C and guanylin reduce fat droplet accumulation in cattle mesenteric adipose tissue

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Guanylyl cyclase C (GC-C) is a member of a family of enzymes that metabolize GTP to cGMP and was first identified as a receptor for heat-stable enterotoxin. Guanylin (GNY) has since been identified as an endogenous ligand for GC-C in the intestine of several mammalian species. The GNY/GC-C system regulates ion transportation and pH in the mucosa. Recently, it was reported that GC-C and GNY are involved in lipid metabolism in rat mesenteric adipose tissue macrophages. To examine the role of GC-C and GNY in lipid metabolism in cattle, we used a bovine mesenteric adipocyte primary culture system and a coculture system for bovine adipocytes and GNY-/GC-C-expressing macrophages. Fat droplets were observed to accumulate in bovine mesenteric adipocytes cultured alone, whereas few fat droplets accumulated in adipocytes indirectly cocultured with macrophages. We also observed that GC-C was present in bovine mesenteric adipose tissue, and that fat droplet accumulation decreased after *in vitro* GNY administration. Expressions of mRNAs encoding lipogenic factors decreased significantly in adipocytes after either coculture or GNY administration. These results suggest that the GNY/GC-C system is part of the control system for lipid accumulation in bovine mesenteric adipose tissue.

Keywords: cattle, guanylin, guanylyl cyclase C, lipogenic factor, mesenteric adipose tissue

Introduction

Guanylyl cyclase C (GC-C) was identified as a receptor for the heat-stable enterotoxin secreted by enterotoxigenic *Escherichia coli* and is abundantly expressed in mammalian intestinal epithelial cells [25,29]. Guanylin (GNY) is mainly present in the intestine and functions as an endogenous ligand of GC-C [5]. GNY binds to the extracellular binding domain of GC-C and activates the intracellular catalytic domain, regulating ion transportation and pH in the mucosa of several mammalian species. In rodents and humans, GC-C and GNY are reportedly expressed in various organs in addition to the intestine, such as adrenal gland, kidney, and ovary [25], suggesting that the GNY/GC-C system has a variety of roles in maintaining homeostasis [4,11,23]. Indeed, it has been reported that GC-C regulates feeding in mice via the central nervous system [10,20,30]. In addition, we have shown that GC-C and GNY are involved in lipid metabolism in rat mesenteric adipose tissue [1]. We also demonstrated that expression of GC-C and GNY by mesenteric adipose tissue macrophages may be related to the high-fat-diet resistant phenotype in rats. In addition, both

GC-C and GNY were expressed only in the mesenteric adipose tissue in the high-fat diet resistant rats. Furthermore, we developed double transgenic (Tg) rats that overexpress both GC-C and GNY and observed that these rats are resistant to the effects of a high-fat diet [1]. Thus, the GC-C/GNY system in rats might be involved in regulating the metabolism of mesenteric adipose tissue.

Currently, little information is available concerning the relationship between GC-C and diarrhea in cattle [3], and it has yet to be determined whether the bovine GNY/GC-C system has a role in regulating energy metabolism. However, it has been shown that an imbalance of adipose tissue metabolism is associated with increased morbidity and mortality rates in this animal [7,27]. In particular, there are some serious bovine production diseases that are related to adipose tissue metabolic diseases such as fat necrosis [16,17]. However, the lipid regulation system of adipose tissues in Japanese black cattle has yet to be fully described. Therefore, to investigate that regulation system, we hypothesized that GNY/GC-C system in cattle has a role in the control of adipose tissue metabolic diseases such as abdominal fat necrosis in Japanese black cattle

[16,17] and performed appropriate experiments as follows. Initially, we investigated the expression of GC-C and GNY in bovine adipose tissues. We used a rat adipocyte culture system when culturing primary bovine mesenteric adipocytes [1,26]. In addition, we used a coculture system for bovine adipocytes and macrophages that express both GC-C and GNY in order to examine how the GNY/GC-C system influences the regulation of adipocytes in cattle. Our results suggest that the GNY/GC-C system in cattle contributes to lipid metabolism in adipose tissue, an outcome that could lead to an improved understanding of the multiple regulatory systems involved in lipid metabolism in Japanese black cattle.

Materials and Methods

Animals

Japanese black calves (n = 6, 1−2 months old) were obtained from local farms in Miyazaki Prefecture, Japan. The calves were euthanized by electric shock following intravenous injection with a combination of xylazine (0.2 mg/kg) and pentobarbital (15 mg/kg). All procedures were performed in accordance with the Japan Physiological Society's guidelines for animal care. This protocol was approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Japan.

Analysis of GC-C and GNY mRNA expression in bovine adipose tissue

Mesenteric adipose tissue samples were collected from the ileal region and RNA was extracted by using an RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). The quality and quantity of RNA were checked by using a spectrophotometer and performing electrophoresis. In addition, expressions of GC-C- and GNY-encoding mRNA were assessed by extracting RNA from the stromal vascular fraction (SVF) and mature adipocytes separated from adipose tissues. The isolation and preservation of SVF samples are described below. Polymerase chain reaction (PCR) primer pairs were designed by using Oligo7 software (Molecular Biology Insights, USA) and are described in Table 1. The PCR products were sequenced by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Japan). The resulting sequencing data indicated that each primer set amplified its target gene precisely (data not shown).

Isolation and preservation of the SVF

Approximately 2 g of mesenteric adipose tissue were collected, washed with Hank's balanced salt solution (HBSS; Invitrogen, USA), minced with scissors, and incubated in a 37°C water bath for 1 h with Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 0.2% type II collagenase (Worthington Biochemical, USA) and 1% bovine serum albumin (Wako, Japan). Mature adipocytes were removed by filtration through a 250 μm mesh. Red blood cells (RBC) were eliminated by a RBC lysis buffer (eBioscience, USA). After centrifugation at 300 × g for 3 min, the sedimented SVF, which contained immature adipocytes, fibroblasts, and macrophages, was collected and preserved in Bambanker cryopreservation medium (Nippon Genetics, Japan) at −80°C

Table 1. Primer pairs used for polymerase chain reaction (PCR) or real-time PCR analysis of fat-maturing factor expression

Primer	Sequence	Mean Ct	Accession number
GC-C forward	ACAAGGCAGAGAGGGACAGG	–	–
GC-C reverse	CAGGCAATAACGAGGCATCT		
GNY forward	AGGAGGAGTCATCGTGAAGGATG	–	–
GNY reverse	CGTAGGCACAGATCTCACACGTG		
PPARγ forward	CATCCGCATCTTCCAGGGGTGTC	25.0	Y12420
PPARγ reverse	GCACGCCGTATTTTAGGAGAGTT		
Fasn forward	AAGCTGCCTGAGTCGGAGAAC	27.2	AY343889
Fasn reverse	CGAAGAAGGAAGCGTCAACC		
Perilipin forward	CCTGGGGGATGGCGAGAGAC	28.6	BC134495
Perilipin reverse	GGCTGGAGGGAGGAGGAACT		
GLUT4 forward	CACTCCTCCTGGGCATCACC	28.4	AY458600
GLUT4 reverse	CAGCTCAGCCAACACCTCAG		
C/EBPα forward	GGTCCCCCTCCTGGCTACGG	29.5	DQ068270
C/EBPα reverse	CGGCGGCTGGTAGGGAAAGA		
GAPDH forward	ACGGCACAGTCAAGGCAGAG	15.5	U85042
GAPDH reverse	CGATGCCAAAGTGGTCATGG		

Ct, threshold cycle; GC-C, guanylyl cyclase C; GNY, guanylin; PPAR, peroxisome proliferator-activated receptors; GULT4, glucose transporter type 4; C/EBPα, ccaat-enhancer-binding protein alpha.

until used.

Culture of primary mesenteric adipocytes

Preserved SVF samples were thawed in a $37^{\circ}C$ water bath for 1 min, washed in HBSS, and then suspended in 10% fetal bovine serum (Equitech-Bio, USA) in DMEM/F-12 + GlutaMax-1 (Invitrogen). Cells were seeded at 1×10^5/well (500 µL) in a 24-well plate (Nunc; Thermo Fisher Scientific, USA) and maintained for a period of 4−5 days at $37^{\circ}C$ in a humidified 5% CO_2 incubator until reaching confluence, during which the culture medium was replaced with fresh medium every 2 days. We confirmed that the time course of differentiation and adipogenesis was similar in cattle and rat adipocytes (data not shown). The culture results showed that adiponectin secretion rapidly increased from day 5 after the beginning of cell culture [26].

Oil Red O staining

Oil Red O staining was performed by using a lipid assay kit (Primary Cell, Japan). Briefly, cells were washed with PBS and fixed with 10% neutral buffered formalin solution (Nacalai Tesque, Japan) overnight at room temperature. The cells were washed again with PBS and stained for 15 min in freshly prepared Oil Red O solution. The stain was removed and the cells were washed 3 times with water and then photographed.

Indirect coculture of primary mesenteric adipocytes and GC-C-/GNY-expressing macrophages

A GC-C-/GNY-expressing rat macrophage cell line (NR8383) was purchased from the American Type Culture Collection (USA) and cultured in F-12 medium (Invitrogen) supplemented with 15% fetal bovine serum (Equitech-Bio). After the primary mesenteric adipocytes became confluent, they were cocultured indirectly with NR8383 cells (1×10^5/well) by using a Millicell cell culture insert (0.4 µm pore size, 12 mm diameter; Millipore, USA) in differentiation medium (Visceral Adipocyte Culture Medium ver. 2 [VACM2]; Primary Cell) for 6 days, during which the culture medium was replaced with fresh medium every 2 days. After the 6 day incubation period, adipocytes (but not NR8383 cells) were harvested and total RNA was extracted by using an RNeasy Plus Micro kit (Qiagen). The primary culture of bovine mesenteric adipocytes was established according to methods described for establishing primary rat adipocytes [1]. The viability of adipocytes was determined by using double staining with calcein-AM and EthD-1 (Live/Dead Viability/Cytotoxicity kit; Invitrogen).

Culture of primary mesenteric adipocytes with GNY

Rat/mouse GNY was purchased from Peptide Institute (Osaka, Japan). Stock solutions of GNY at various concentrations (100 nM, 500 nM, 1 µM, and 5 µM) were prepared in differentiation medium (VACM2; Primary Cell). Primary mesenteric adipocytes were cultured in the presence of GNY for 6 days, during which the culture medium was replaced with fresh medium every 2 days. After the 6 day incubation period, the medium was removed and total RNA was extracted by using an RNeasy Plus Micro kit (Qiagen).

Quantitative PCR

The number of mRNA transcripts corresponding to the various adipocyte maturation and lipogenesis genes expressed in mesenteric adipocytes was determined by performing quantitative PCR. Real-time PCR primer pairs were designed by using Oligo7 software (Molecular Biology Insights) and are described in Table 1. First-strand cDNA was synthesized from 1 µg total RNA by using a commercially available Superscript III First-Strand Synthesis System kit (Invitrogen), and the resulting samples were subjected to quantitative PCR. Real-time PCR was conducted by using a LightCycler system (Roche Diagnostics, Germany) and an Ex Taq II SYBR premix system (TaKaRa Bio, Japan). The level of mRNA expression of each target gene was normalized against that of GAPDH. The gene expression levels of GAPDH in each sample were not different, therefore we determined that GAPDH could be used as the reference gene for real-time PCR. The data were analyzed by using LightCycler software version 3.5 (Roche Diagnostics).
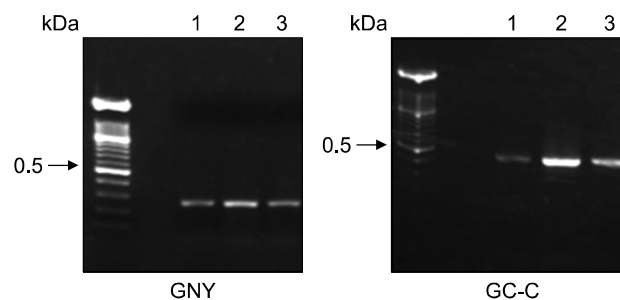
Statistical analysis

Data were analyzed by undertaking analysis of variance and the *post hoc* Tukey-Kramer test. A p value < 0.05 was considered significant. Data are presented as mean ± SEM values.

Results

Expression of mRNAs encoding GC-C and GNY in bovine adipose tissue

Fig. 1 presents images showing the expressions of mRNAs

Fig. 1. Expression of guanylyl cyclase C (GC-C) and guanylin (GNY) mRNAs in bovine cultured adipocytes (lane 3), stromal vascular fraction (lane 2), and mesenteric adipose tissue (lane 1).

encoding GC-C and GNY in cultured bovine adipocytes, SVF, and mesenteric adipose tissue. All samples examined showed expression of the GC-C- and GNY-encoding mRNAs.

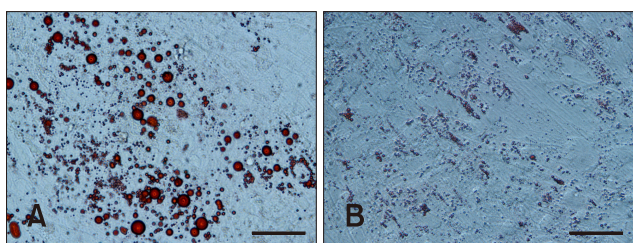### Indirect coculture of primary mesenteric adipocytes with NR8383 cells

Individual fat droplets stained with Oil Red O appeared red in color. The primary culture of bovine mesenteric adipocytes was established according to methods used for establishing primary rat adipocytes, and numerous large fat droplets were observed after 6 days of *in vitro* culture (panel A in Fig. 2). However, in primary mesenteric adipocytes cocultured with NR8383 cells, only a few small fat droplets were observed (panel B in Fig. 2). In addition, the viability of adipocytes cocultured with NR8383 cells was very high (data not shown).

Culture of primary mesenteric adipocytes with GNY

As determined by Oil Red O staining, the number and size of fat droplets in primary mesenteric adipocytes cultured in the presence of rat GNY decreased with increasing GNY concentration, as shown in panels A−E in Fig. 3. High concentrations of GNY had a particularly marked inhibitory effect on the formation of fat droplets in primary mesenteric adipocytes. In addition, the viability of adipocytes during culture with each concentration of GNY was more than 90% (data not shown).

mRNA expression

Significantly reduced expressions of mRNAs encoding perilipin ($p < 0.0005$ *vs*. control cells), Fasn ($p < 0.05$), and ccaat-enhancer-binding protein (C/EBPα; $p < 0.05$) were observed in cocultured adipocytes (Fig. 4). In adipocytes cultured with GNY, expression of mRNAs encoding peroxisome proliferator-activated receptors (PPARγ), perilipin, glucose transporter type 4 (GLUT4), and Fasn decreased with an increase in the concentration of GNY (Fig. 5). Expression of

C/EBPα-encoding mRNA declined significantly ($p < 0.05$) in cells cultured with 5 μM GNY.

Discussion

In rats and humans, GNY and GC-C are present mainly in the intestine, where they function in maintaining homeostasis of body fluids [8,9,13,14,19,25,31]. It was recently reported that overexpression of GC-C and GNY in macrophages has a role in regulating lipid metabolism [1]. Previously, we demonstrated that the GNY and GC-C system in rat macrophages regulates mesenteric fat inflammation [15]. Several reports on immune responses in cattle adipose tissue have been published [2,22]. The infiltration of phagocytic cells into bovine adipose tissue does not have a major role in immunologic and metabolic adaptations in dairy cows. This during early lactation [2]. However, some monocyte chemoattractant protein gene expressions increase after induction of inflammation [22]. In mesenteric adipose tissue of Japanese black calves (1−2 months old), some immunological cells such as a few CD4+ cells and MHC class II+ cells have been observed (Yasuda *et al*., manuscript in preparation). Therefore, immune responses in
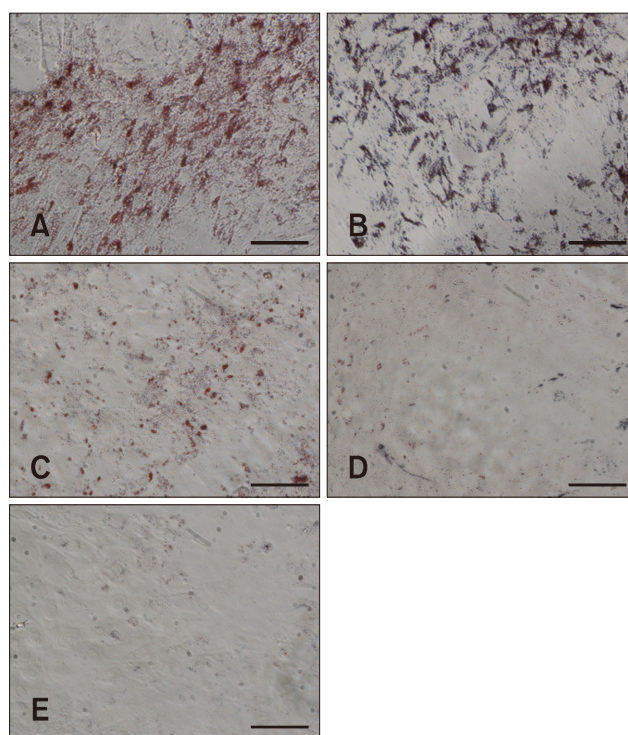

Fig. 2. Oil Red O staining of primary mesenteric adipocytes cultured with guanylyl cyclase C (GC-C)-/guanylin (GNY)-expressing macrophages. (A) Numerous large fat droplets were observed in primary mesenteric adipocytes. (B) The number of fat droplets declined in primary mesenteric adipocytes cocultured with macrophages. Few fat droplets were observed in the primary mesenteric adipocytes. Scale bars = 50 μm.
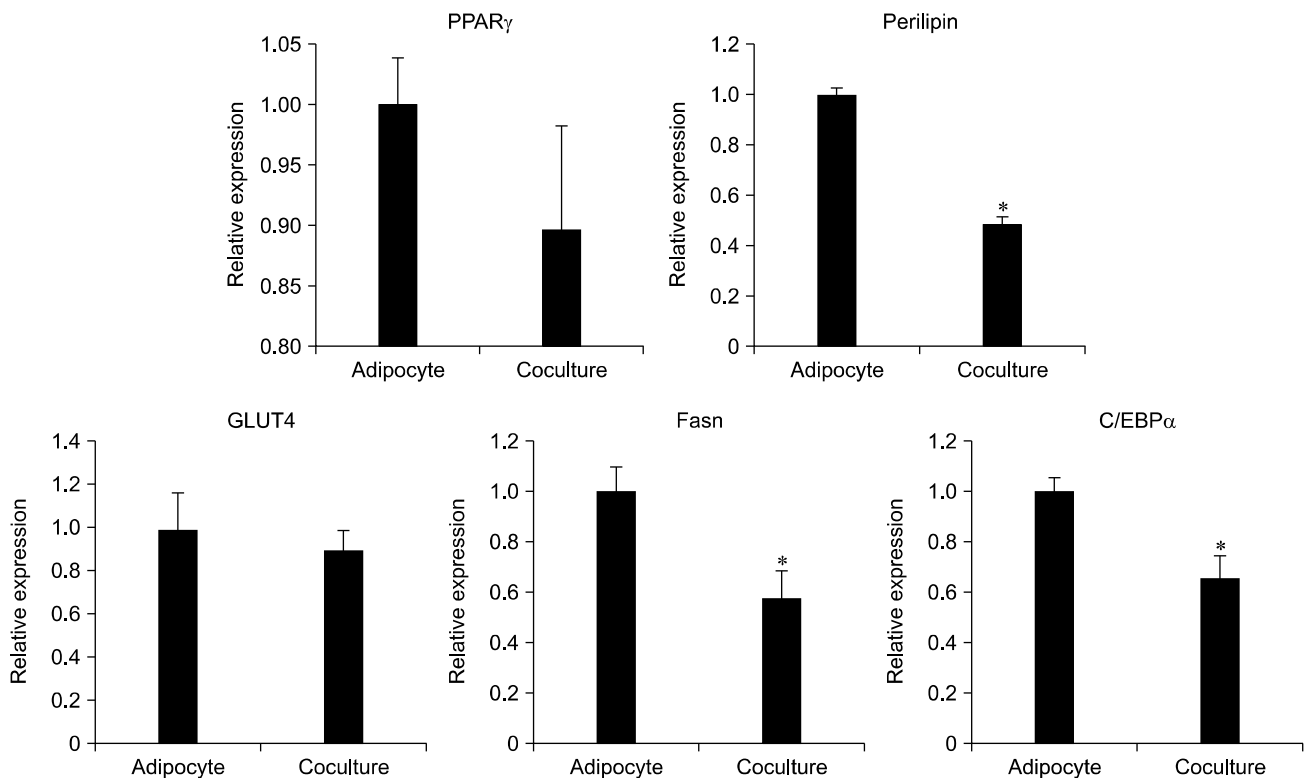

Fig. 3. Oil red O staining of primary mesenteric adipocytes cultured with varying concentrations of rat guanylin (GNY). No GNY (A), 100 nM GNY (B), 500 nM GNY (C), 1 μM GNY (D), or 5 μM GNY (E) added to the culture medium. The number of fat droplets in the primary mesenteric adipocytes decreased with an increased concentration of rat GNY. Scale bars = 50 μm.

Fig. 4. Expression of mRNAs encoding various fat-maturing factors in primary mesenteric adipocytes indirectly cocultured with guanylyl cyclase C (GC-C)-/guanylin (GNY)-expressing macrophages. Expression of mRNAs encoding the indicated factors was lower in primary mesenteric adipocytes cocultured with GC-C-/GNY-expressing macrophages. Data are presented as mean ± SEM values. Asterisks denote significant differences ($p < 0.05$).

adipose tissue might differ with cattle strain, age, and/or health condition. Further analysis is needed to evaluate the immune response of adipose tissue in cattle.

In our analysis of a new pathway for lipid metabolism in bovines, we focused on the evaluation of a GNY/GC-C system in cattle adipocytes. The results show that both GC-C and GNY are expressed in bovine mesenteric fat tissue, suggesting that the GNY/GC-C system may have a role in regulating lipid metabolism in cattle. Furthermore, in this study, we developed a primary culture system for bovine adipocytes by applying an established rat culture system. Considering the practical difficulties of producing and evaluating transgenic or knockout cattle, the establishment of this culture system is useful for elucidating the mechanism of bovine lipid metabolism.

In order to examine the practical contribution of the GNY/GC-C system to lipid metabolism in cattle, we used a system involving indirect coculture of bovine adipocytes and NR8383 macrophages that express both GC-C and GNY. The results demonstrate that lipid accumulation in bovine adipocytes is significantly inhibited following coculture with NR8383 cells. In addition, we examined the mRNA expression of fat differentiation, maturation, and lipogenesis factors such as PPARγ, C/EBPα, GLUT4, Fasn, and perilipin. Generally,

PPARγ and C/EBPα are involved in adipocyte differentiation [6,24], whereas GLUT4, an insulin-dependent glucose transporter, is recognized as a maturation factor [12,32]. Perilipin has a role in droplet formation [28], and Fasn is essential for lipogenesis and generation of the palmitic acid component of fatty acids [21]. The results of the present study showed that expression of mRNAs encoding C/EBPα and perilipin decreased significantly in adipocytes following coculture with NR8383 cells. Expression of the mRNA encoding Fasn also decreased following coculture. These results are similar to those obtained from coculture of peritoneal macrophages of GNY/GC-C Tg rats and rat mesenteric adipocytes [1]. Thus, our results suggest that the GNY/GC-C system in mesenteric fat tissues of both cattle and rats regulates lipid accumulation, resulting in control of lipogenesis.

The present study showed that GNY and GC-C are expressed in both SVF and mature adipocytes in bovine adipose tissues, and, when bovine adipocytes were cultured with GNY, lipid accumulation was inhibited. In contrast, GC-C is not expressed in rat adipocytes, and when rat adipocytes were cultured with GNY, lipid accumulation was not inhibited [1]. These findings suggest that there are differences in the cellular source of GNY and GC-C in the adipose tissue of these two species. To
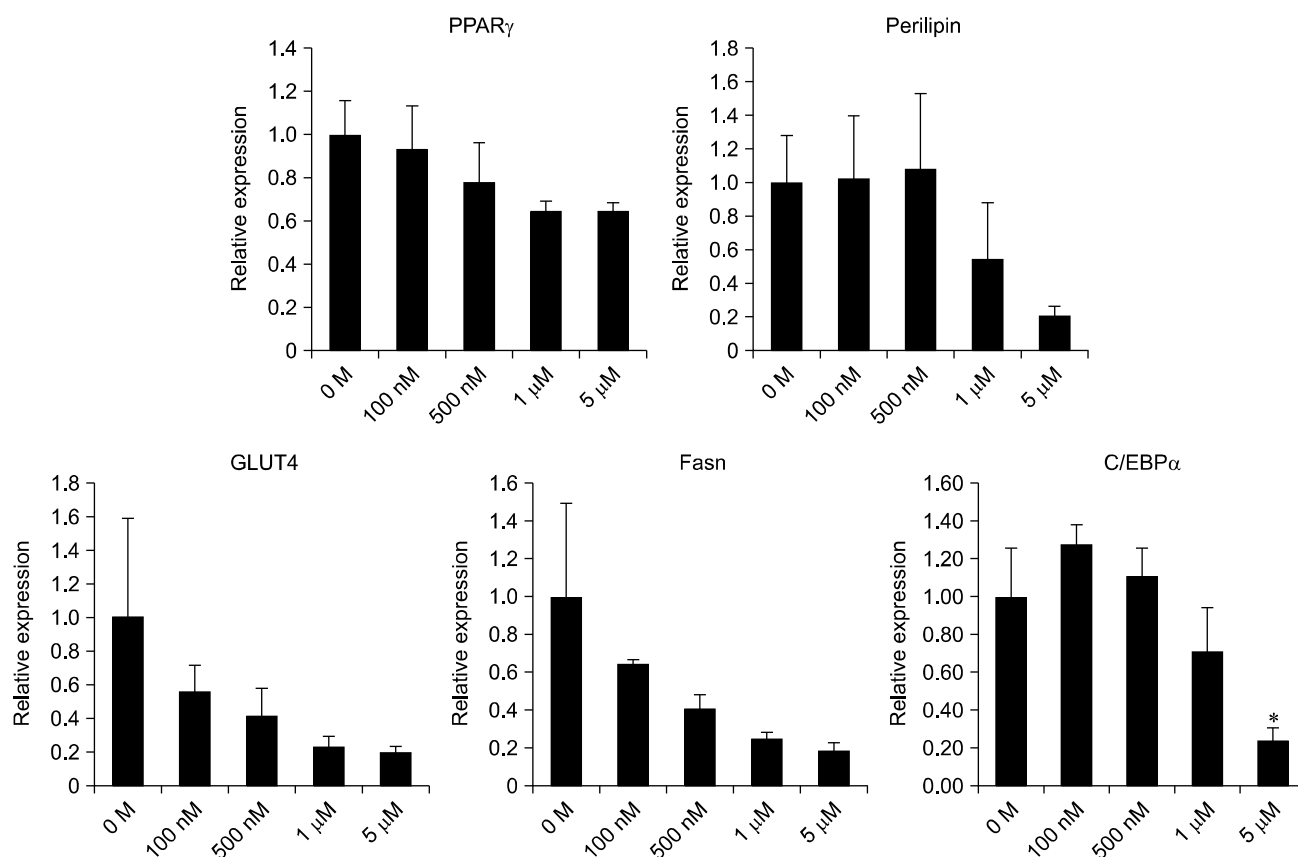
Fig. 5. Expression of mRNAs encoding various fat-maturing factors in primary mesenteric adipocytes cultured in the presence of various concentrations of rat guanylin (GNY). Expression of mRNAs encoding the indicated factors decreased with an increased concentration of rat GNY. Data are presented as mean $\pm$ SEM values. Asterisk denotes a significant difference ($p < 0.05$ *vs*. 0 M and 100 nM).

elucidate the mechanism of GNY/GC-C action for lipid regulation, identification of the cellular source of GNY and GC-C in the adipose tissue of different species is required. In this study, we also showed that expressions of the mRNAs encoding PPARγ, perilipin, GLUT4, and Fasn decreased in the presence of GNY in a dose-dependent manner. In addition, we observe that expression of C/EBPα mRNA declines significantly when adipocytes are cultured in a medium containing a high concentration of GNY. As we used rat/mouse GNY in our experiments, a high GNY concentration might have been needed to detect the inhibition of lipid accumulation or lipogenic factors. We have already cloned bovine GNY and observed that the homology between the coding sequences of rat and bovine GNY is about 70% (Yasuda *et al*., manuscript in preparation). Therefore, we hypothesize that bovine GNY may have a greater effect than rat/mouse GNY on metabolism in bovine mesenteric adipose tissues. Taken together, our results indicate that in cattle, GC-C and GNY in both macrophages and adipocytes have roles in lipid metabolism. To clarify how the interaction of GNY-/GC-C macrophages and adipocytes function to regulate lipid metabolism in cattle, more

investigation is required. In addition, in cattle, the secretion of adipokines including adiponectin from adipose tissues has a role in lipid metabolization [18]. Therefore, it is also important to analyze further the relationship of the cattle GNY/GC-C system with other lipid metabolization systems.

In this study, we investigated the association between lipid accumulation and the GNY/GC-C system in macrophages by using an indirect coculture approach. The accumulation of lipid was clearly inhibited after indirect coculture, suggesting that the macrophage GNY/GC-C system induces the secretion of soluble factors that are involved in regulating lipid accumulation in cattle. Identification of the soluble factors produced by GNY-/GC-C-expressing macrophages will provide new insights into the regulation of lipid metabolism in cattle.

Acknowledgments

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Conflict of Interest

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