ORIGINAL ARTICLE

Glucose enhances catecholamine-stimulated lipolysis via increased glycerol-3-phosphate synthesis in 3T3-L1 adipocytes and rat adipose tissue

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Abstract

Background During lipolysis, triglyceride (TG) are hydrolyzed into a glycerol and fatty acids in adipocyte. A significant portion of the fatty acids are re-esterificated into TG, and this is a critical step in promoting lipolysis. Although glycerol-3-phosphate (G3P) is required for triglyceride synthesis in mammalian cell, the substrate for G3P synthesis during active lipolysis is not known. A recent study showed that the inhibition of glucose uptake reduces catecholamine-stimulated lipolysis, suggesting that glucose availability is important in lipolysis in adipocytes. We hypothesized that glucose might play an essential role in generating G3P and thereby promoting catecholamine-stimulated lipolysis in adipocytes. Therefore, we determined the effect of glucose availability on catecholamine-stimulated lipolysis in 3T3-L1 adipocytes and rat adipose tissue.
Methods and Results 3T3-L1 adipocytes and rat epididymal fat pads were cultured in a medium with/without glucose during stimulation by isoproterenol. Glycerol release was higher when adipocytes were cultured in a glucose-containing medium than that in a medium without glucose. Measurement of glucose uptake during catecholamine-stimulated lipolysis showed a slight, but significant increase in glucose uptake. We also compared glucose metabolism-related protein, such as glucose transporter 4, hexokinase, glycerol-3-phosphate dehydrogenase and lipase contents between fat tissues that play a critical role in active lipolysis. Epididymal fat exhibited higher lipolytic activity than inguinal fat because of higher lipase and glucose metabolism-related protein contents.
Conclusion We demonstrated that catecholamine-stimulated lipolysis is enhanced in the presence of glucose, and suggests that glucose is one of the primary substrates for G3P in adipocytes during active lipolysis.

Keywords Glycerol · Fat · Glucose uptake · Isoproterenol

Introduction

Triglyceride (TG) is a major form of lipid storage in adipose tissue. Glycerol-3-phosphate (G3P) and fatty acyl-CoA are the substrate for TG synthesis in mammalian cells. G3P can be generated by the reduction of dihydroxyacetone phosphate, which is derived from either glucose or pyruvate via glycolysis or glyceroneogenesis, respectively. G3P can also be generated by the direct phosphorylation of glycerol by glycerol kinase but this alternative pathway is less active in adipose tissue [1]. Thus, glucose and pyruvate are the main carbon sources for G3P synthesis in adipose tissue.

Lipolysis in adipose tissue is stimulated by catecholamine to release fatty acids (FA) into the blood stream, and these FAs are used as an energy source in the skeletal muscle and liver during exercise and fasting [2]. During active lipolysis, about two-thirds of FAs that are obtained by TG hydrolysis are re-esterified to form TG [3, 4]. The significance and mechanism of this re-esterification step was unclear for decades; however, recent reports showed that this step is critical for facilitating lipolysis in adipocytes. A report showed that the inhibition of re-esterification of FAs into TG decreased the number of micro-lipid droplets and the lipolytic activity in 3T3-L1 adipocytes [5].

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For the re-esterification of FA, G3P is required to be generated from precursors such as glucose and pyruvate. However, the culture media used in some studies pertaining to lipolytic activity examination were devoid of any of these substrates [6–9]. Moreover, our previous study showed that the inhibition of glucose transport by cytochalasin B caused a decrease in catecholamine-stimulated lipolysis in adipocytes, which were cultured in a glucose-containing medium [10]. This result led us to consider that glucose might play an important role in generating G3P and thereby promoting catecholamine-stimulated lipolysis in adipocytes. Therefore, in the present study, we determined the effect of glucose availability on catecholamine-stimulated lipolysis in 3T3-L1 adipocytes and rat adipose tissue.

Methods and materials

Materials

All reagents for cell culture were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. Animal studies were performed according to national guidelines for the care and use of laboratory animals (Notification of the Prime Minister's Office of Japan).

Treatment of animals

Animal Experimental Committee of University of Shiga Prefecture approved this experimental protocol (Ref #30-10). Four-week-old male Wistar rats were obtained from CLEA Japan, Tokyo, Japan). The animals were maintained under a constant light/dark cycle (light from 6 a.m. to 6 p.m.) and given free access to food and water.

Adipose tissue dissection and incubation

Rats were anesthetized using isoflurane, and inguinal and epididymal adipose tissues were removed as subcutaneous and abdominal fats, respectively. One portion of fat pads was quickly frozen in liquid nitrogen for determination of protein content by western blot analysis. Remaining tissues were cut into pieces of 30 mg weight and were then cultured into 500 µL of glucose-free, phenol red-free Dulbecco's modified Eagle's medium (DMEM) containing 2% (w/v) FA-free bovine serum albumin (BSA) in a 24-well plate and in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell culture and lipolytic stimulation

3T3-L1 cells were cultured according to a previous report [10]. Lipolytic stimulation of differentiated 3T3-L1 adipocytes and rat fat pads was performed according to the following protocol: 3T3-L1 adipocytes were washed with phosphate buffer saline (PBS), then cultured in phenol red-free DMEM containing 2% BSA with 1 µM of isoproterenol (Iso) in presence or absence of 5.5 mM glucose at 37 °C for 4 h. Rat fat pads were cultured in either phenol red-free DMEM (containing 2% BSA) or Krebs–Ringer bicarbonate HEPES buffer (pH 7.4, 2% BSA, 120 mM NaCl, 30 mM HEPES, 10 mM sodium bicarbonate, 4 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) with 1 µM of Iso in presence or absence of 5.5 mM glucose at 37 °C for 2 h. For a dose response experiment for glucose, 3T3-L1 adipocytes were incubated with DMEM containing either 0 mM, 2.3 mM, 5.5 mM or 11 mM glucose in the presence of 1 µM isoproterenol for 4 h. Mannitol was used as an osmotic control in a non-substrate containing medium. Propranolol (1 µM) was added to the medium 15 min before lipolysis stimulation. Aliquots of the medium were collected and glycerol and glucose levels were measured using a glycerol assay kit (Sigma, MO) and a Glucose CII test Wako kit (Fujifilm, Japan), respectively.

Glucose uptake

3T3-L1 adipocytes were grown in 6-well plates as described above. The cells were washed 3 times with PBS and incubated in phenol red-, glucose-free DMEM containing 2% BSA and 1 µM isoproterenol in the presence or absence of 2-deoxy-glucose (2DG) and glucose (1:1 molar ratio) for 2 h at 37 °C. Our preliminary experiment has shown that when adipose tissue is cultured with 2DG, glycerol release is lower than when cultured with glucose (Glucose containing medium; 10.1 ± 0.8 nmol/mg tissue, 2DG containing medium; 6.1 ± 0.3 nmol/mg tissue, $n = 5$, $p < 0.01$). Therefore, we used a mixture of 2DG and glucose to measure the 2DG uptake rate during catecholamine-stimulated lipolysis.

Western blot analysis

3T3-L1 adipocytes and rat fad pads were homogenized in an ice-cold radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and protease and phosphatase inhibitor cocktail. The homogenates were kept on ice for 10 min, and then were centrifuged at 15,000 g for 10 min. The supernatants were collected, and protein levels were measured using a bicinchoninic acid assay kit. Samples were prepared in the 4X Laemmli sample buffer. Equal amounts of sample proteins were separated using 7.5% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Pierce Biotechnology, IL), and incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: anti-adipose triglyceride

lipase (ATGL) (2138), anti-hormone sensitive lipase (HSL) (4107), anti-phospho-protein kinase A (PKA) substrate (9624), anti-glucose transporter (GLUT)4 (2213), anti-hexokinase (HK)2 (2160) and all primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-glycerol-3-phosphate dehydrogenase (GPD1) (13451-1-AP) antibody was purchased from Proteintech (Rosemont, IL), anti-phosphoenolpyruvate carboxykinase (PEPCK) (sc-32879) and anti-comparative gene identification-58 (CGI-58) (sc-102285) antibodies were obtained from Santa Cruz Technology (Santa Cruz, CA). The protein bands were detected by enhanced chemiluminescence (Merck, Darmstadt, Germany). Images were scanned using a chemiluminescence detector (LAS500, GE Healthcare Bio-Sciences AB, Upsala, Sweden). Band intensities were quantified using ImageJ 1.52a (National institutes of Health, Bethesda, ME). Loading of proteins in equal amounts was ensured by staining the blot with Coomassie Brilliant Blue (CBB) (Wako, Osaka, Japan) [11].

Biochemical analyses

For measuring the levels of cellular metabolites, 3T3-L1 adipocytes were treated as described under "Lipolytic stimulation." After incubation, the cells were washed twice with cold PBS, and harvested using 0.1 N HCl. After neutralization with 0.1 N NaOH, the samples were centrifuged at 3000 g for 10 min, and the supernatants were used to measure the levels of cellular G3P and 2DG6P. The G3P level was measured using commercial kits (AAT Bioquest, Sunnyvale, CA). The 2DG6P level was measured as described previously [12].

Statistical analysis

Data are expressed as means ± standard errors of the mean. Statistical analyses were performed using BellCurve for Excel version 3.21 (Social Survey Research Information, Tokyo, Japan). Differences between groups were assessed by one- or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, or unpaired t-tests, as indicated. A p value of < 0.05 was considered statistically significant.

Results

Lipolytic activity is enhanced in the presence of glucose in 3T3-L1 adipocytes and rat adipose tissue

As shown in Fig. 1A, basal lipolysis was not affected by the presence of glucose. Stimulation by isoproterenol resulted in an increase in glycerol release into the culture medium in the absence of glucose. In the presence of glucose at a concentration of 5.5 mM, glycerol release was increased by twofold compared with that in the absence of glucose (Fig. 1B, C). This effect of glucose on increased glycerol release was also observed in rat epididymal adipose tissues incubated with DMEM (Fig. 1D–F) or KRBH buffer (Fig. 1G–I). The FFA levels in the medium was increased by isoproterenol in 3T3-L1 adipocytes and rat adipose tissue, but it was decreased in the presence of glucose (Fig. S1). We also found that the presence of 5.5 mM glucose in the medium become saturating (Fig. S2). These results indicate that the presence of glucose in a culture medium enhances lipolytic activity in 3T3-L1 adipocytes and rat adipose tissue.

Increase in lipolysis in the presence of glucose is because of the higher provision of G3P

De novo synthesis of G3P from pyruvate called glyceroneogenesis is considered a potential pathway for TG synthesis in adipose tissue [13, 14]. Next, we incubated adipocytes with glucose or pyruvate to determine their lipolysis-enhancing effect. The lipolysis-enhancing effect was observed only in the 3T3-L1 adipocytes cultured in the glucose-containing medium (Fig. 2A). Catecholamine stimulates the protein kinase A signaling pathway and activates downstream lipases including ATGL [15, 16]. To determine whether glucose affects these signaling pathway during lipolysis, we measured the phosphorylation levels of PKA substrates. After stimulation with 1 µM isoproterenol, the phosphorylation levels of PKA substrates increased about threefold regardless of type of substrates (Fig. 2B). Furthermore, the G3P level in adipocytes was higher in the presence of glucose than that in the presence of mannitol and pyruvate. These results suggest that the enhancement of catecholamine-stimulated lipolysis in the presence of glucose is because of the higher provision of G3P and not because of the activation or inactivation of lipolysis-related signaling pathway.

Lipolytic stimulation increases glucose uptake in 3T3-L1 adipocytes

Although the insulin stimulated-glucose uptake in adipocytes has been extensively studied, the effect of catecholamine stimulation on glucose uptake in adipocytes is not well examined. To confirm the results that the presence of glucose increases catecholamine-stimulated lipolysis in adipocytes was because of an increase in the G3P level, we measured the glucose uptake rate under lipolytic stimulation. In the presence of isoproterenol, glucose concentration in culture medium tended to lower compared with that in the absence of isoproterenol, although not statistically significant (Fig. 3A). In addition, glucose uptake in 3T3-L1
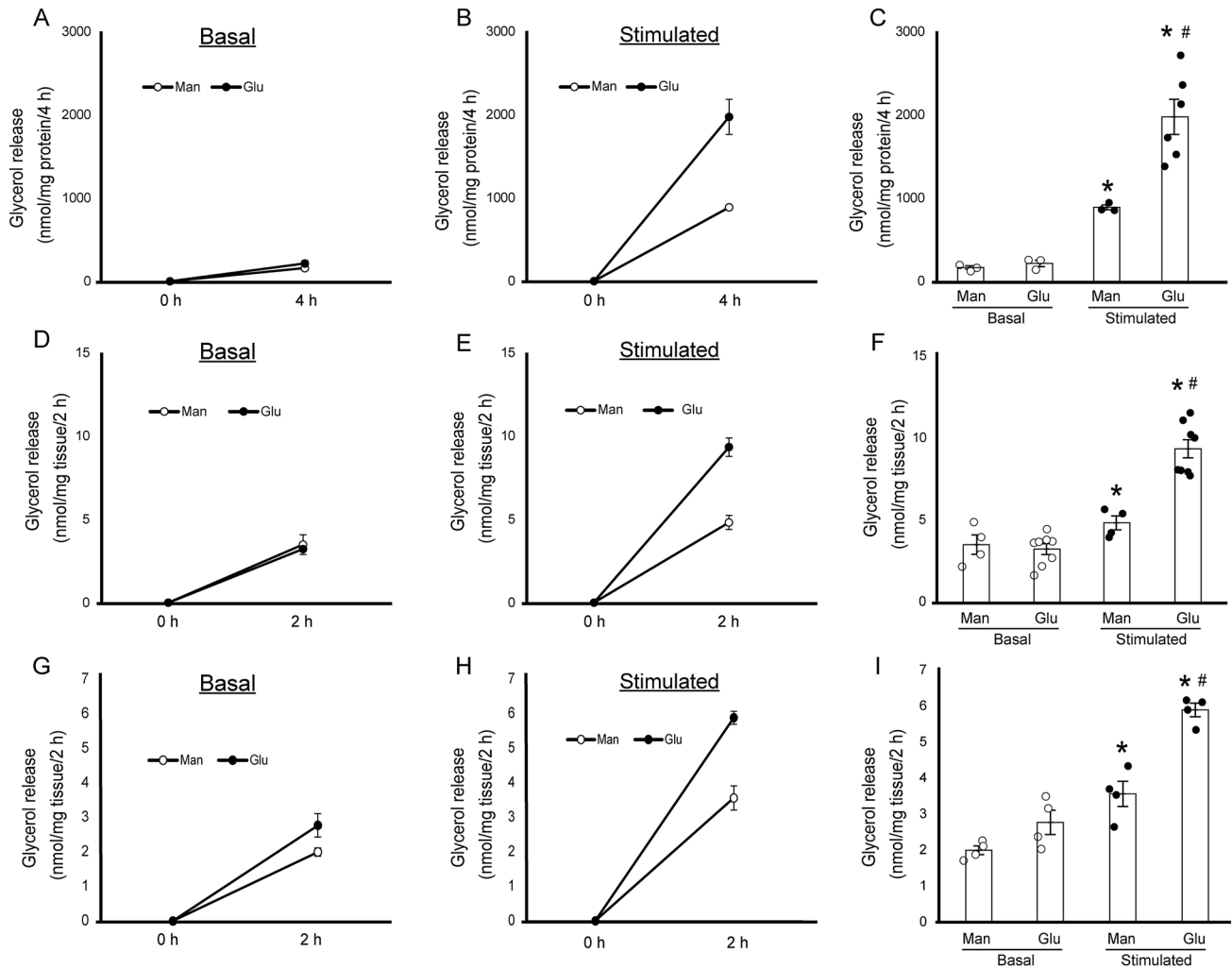
Fig. 1 Effect of glucose on lipolysis in basal and catecholamine-stimulated state. 3T3-L1 adipocytes (**A**–**C**) were cultured in DMEM with 1 μM of isoproterenol (Iso) with/without 5.5 mM glucose for 4 h. Rat adipose tissues were cultured in either DMEM (**D**–**F**) or Krebs–Ringer bicarbonate HEPES buffer (**G**–**I**) with 1 μM of Iso with/without 5.5 mM glucose for 2 h. The release of glycerol during basal (**A**, **D**, **G**)- and Iso (**B**, **E**, **H**)-stimulated lipolysis were measured after stimulation with or without Iso. All data are presented as means $\pm$ SEM. $n = 3$–8. Two-way ANOVA was performed. *$p < 0.05$ vs. same treatment in basal group. #$p < 0.05$ vs. Man group. *Man* Mannitol, *Glu* Glucose

adipocytes was accelerated by isoproterenol stimulation, as measured by the accumulation of 2DG6P (Fig. 3B). Furthermore, the effect of catecholamine on glucose uptake was blocked by pretreatment with beta-adrenaline receptor antagonist, propranolol. These results indicate that lipolytic stimulation increases glucose uptake in adipocytes.

Glucose metabolism-related protein contents were higher in intra-abdominal fat than in subcutaneous fat

Higher catecholamine-stimulated lipolytic activity has been reported in intra-abdominal fat than that of subcutaneous fat in previous studies. This difference is because of the higher expression of lipases [7] or sensitivity of

receptors in intra-abdominal fat [17]. We compared glucose metabolism-related protein and lipase contents between fat tissues that play a critical role in active lipolysis. As shown in Fig. 4, epididymal fat exhibited higher levels of lipases such as ATGL and HSL compared with inguinal fat, whereas the level of CGI-58, a coactivator of ATGL, was the same in both the fat cells. In addition, epididymal fat exhibited higher levels of glucose metabolism-related proteins such as GLUT4, HK2, and GPD1 than inguinal fat. The protein content of PEPCK, a rate-limiting enzyme involved in producing G3P from pyruvate, was similar in both the fat cells. These results indicated that not only lipolytic enzymes but also glycolytic protein contents contribute to higher lipolytic capacity in epididymal fat than in inguinal fat.
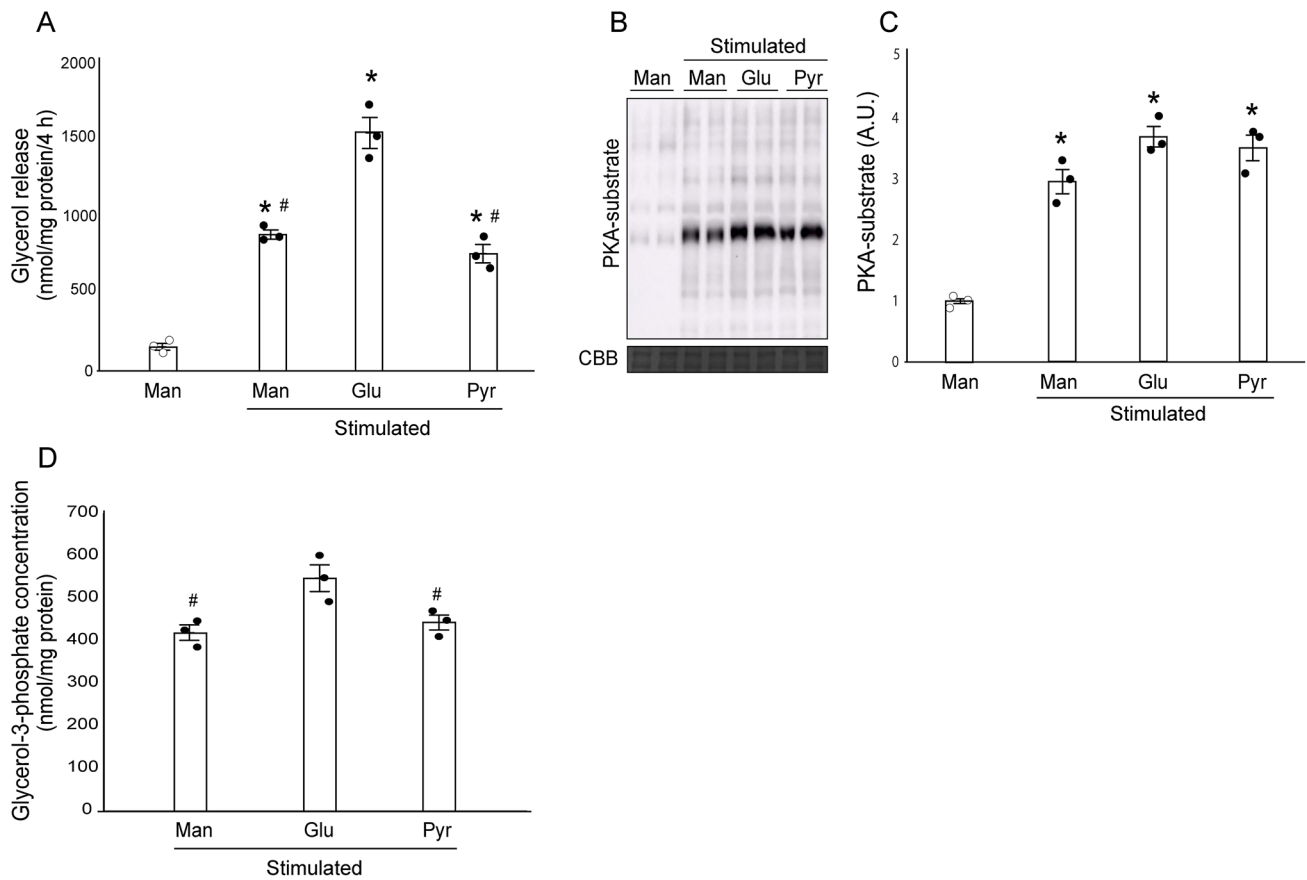
Fig. 2 Glucose, not pyruvate enhances catecholamine-stimulated lipolysis in adipocytes. 3T3-L1 adipocytes were stimulated by 1 μM of isoproterenol (Iso) in the DMEM containing either 5.5 mM mannitol, glucose or pyruvate. **A** Glycerol concentration in medium after stimulation with Iso for 4 h. Representative blot (**B**) and quantitation (**C**) of PKA substrate after catecholamine stimulation. Coomassie brilliant blue (CBB) staining serves as a loading control. G3P concentration in adipocytes after 1 μM Iso treatment for 4 h (**D**). All data are presented as means ± SEM. $n = 3$. One-way ANOVA was performed. *$p < 0.05$ vs. unstimulated-Man group. #$p < 0.05$ vs. stimulated-Glu group. *Man* Mannitol, *Glu* Glucose, *Pyr* Pyruvate

Fig. 3 Glucose uptake is slightly, but significantly increased by catecholamine stimulation in adipocytes. **A** Glucose concentration during lipolysis stimulation by 1 μM of isoproterenol for 2 h. **B** 2-deoxy-glucose uptake during 2-h catecholamine-stimulated lipolysis in 3T3-L1 adipocytes. All data are presented as means ± SEM. $n = 6$–9. One-way ANOVA was performed. *$p < 0.05$ vs. basal group. #$p < 0.05$ vs. Iso + propranolol group
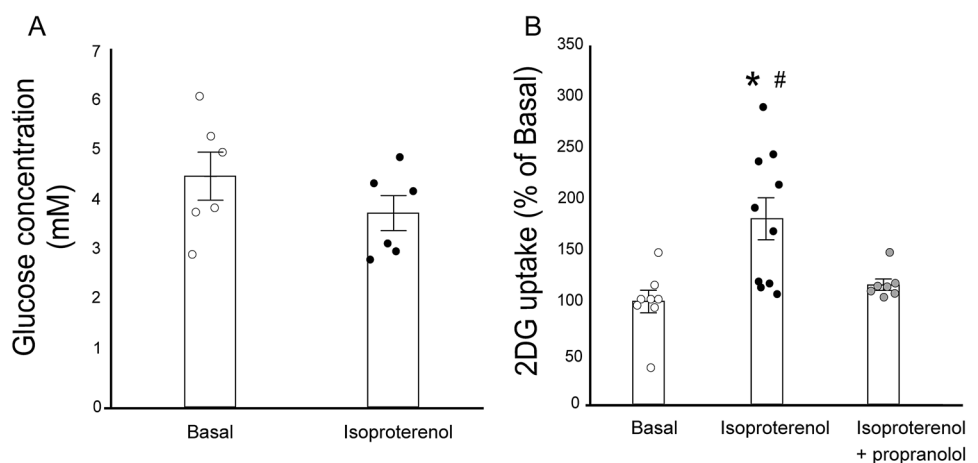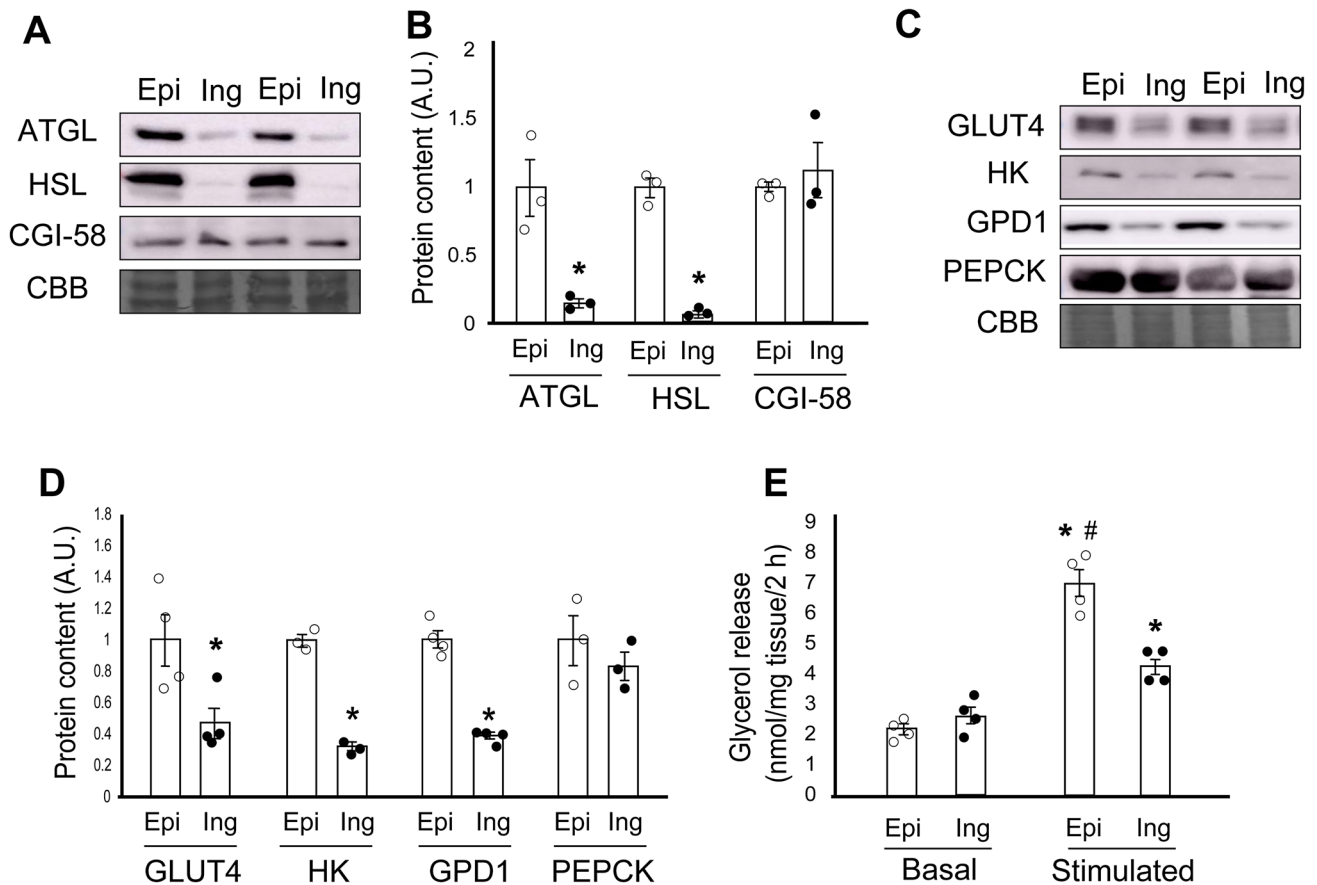
Fig. 4 Lipases and glucose metabolism-related proteins expression in rat epididymal and inguinal adipose tissue. Representative blots (**A**) and quantitation (**B**) of ATGL, HSL, and CGI-58 in epididymal and inguinal adipose tissue. Representative blots (**C**) and quantitation (**D**) of GLUT4, HK2, GPD1, and PEPCK in epididymal and inguinal adipose tissue from rat. Coomassie brilliant blue (CBB) staining serves as a loading control. Data are presented as means ± SEM. $n = 3$–4.

Unpaired Student's t-test was performed. $*p < 0.05$ vs. inguinal fat. Basal and Iso-stimulated lipolysis were measured as the release of glycerol after stimulation with or without Iso in glucose-containing medium (**E**). Data are presented as means ± SEM. $n = 3$–4. Two-way ANOVA was performed. $*p < 0.05$ vs. Basal condition. $\#p < 0.05$ vs. Ing in stimulated condition

Discussion

Understanding the underlying mechanism by which glucose availability controls this process is essential. Therefore, we aimed to elucidate an underlying mechanism by which glucose participates in the re-esterification of FA and promotes active lipolysis in adipocytes. TGs are synthesized in adipocytes during lipolysis by re-esterification. In our study, adipocytes exhibited a higher lipolytic activity in a glucose-containing medium than no-glucose or pyruvate-containing medium, suggesting that glucose and not pyruvate is the primary substrate in G3P synthesis during catecholamine-stimulated lipolysis.

Our results showed that catecholamine stimulation increases glucose uptake in adipocytes. Previous studies reported a slight increase in the glucose uptake rate when adipocytes were stimulated by catecholamine [18, 19]. Smith et al. reported a twofold increase in the 3-O-methylglucose

transport activity induced by isoproterenol in rat primary adipocytes, whereas an increase by 20-fold was reported by insulin stimulation [19]. A recent study reported a slight increase in glucose uptake induced by catecholamine and inhibited by beta-adrenaline receptor antagonist in mouse primary adipocytes [18]. Therefore, our results are in accordance with the previous studies indicating that the increase in glucose uptake by catecholamine promotes lipolysis by acting as the primary substrate for G3P generation in adipocytes.

Intra-abdominal fat exhibits a higher lipolytic capacity than that of subcutaneous fat because of the difference in levels of lipolytic enzymes expressed by them [7]. Similarly, we found higher levels of ATGL and HSL in epididymal fat than in subcutaneous fat (Fig. 4A, B). In addition to lipases, the levels of GLUT4, HK2, and GPD1, which synthesizes G3P from dihydroxyacetone phosphate, were higher in epididymal fat than those in inguinal fat (Fig. 4C,

D). In contrast, the expression of PEPCK, a key enzyme for glyceroneogenesis did not differ between both the fats. Results from our study and previous studies indicate that not only lipase expressions, but also glucose availability plays an essential role in catecholamine-stimulated lipolysis in adipose tissue.

Transport of glucose across the cell membranes is mediated by members of glucose transporter GLUT [20, 21]. Basal glucose uptake is mediated by GLUT1 in many cells [22]. A reason for the slight increase in glucose uptake during lipolysis observed in the present study could be the changes in the intrinsic activity of GLUT1 induced by catecholamine. Insulin stimulation causes translocation of GLUT4 from intracellular pool to plasma membrane, and a concomitant increase in glucose uptake [23, 24]. However, a previous study reported that the activation of cAMP-dependent protein kinase signaling does not cause translocation of GLUT4 in adipocytes [25]. This shows that a slight increase in glucose uptake by catecholamine stimulation is not because of the increase in GLUT4 abundance in the plasma membrane. While our study was in progress, Beg M et al. [26] reported that inhibition of ATGL by atglistatin completely blocked the catecholamine-stimulated glucose uptake in adipocytes. Therefore, not only activation of adrenalin receptor, but also activation of the downstream pathway such as ATGL play important roles in the upregulation of catecholamine-stimulating glucose uptake in adipocytes. The modification of intrinsic activities and expression levels of these two glucose transporters must be evaluated in a future study.

It is reported that many factors influence lipolytic activity in adipocyte although we have not examined in this study. For instance, adenosine is known to inhibit lipolysis by suppression of cAMP, and addition of adenosine deaminase to culture medium improves catecholamine-stimulated lipolysis [27]. Further studies are required to examine the effect of glucose availability on the efficacy of pharmacological reagents that activate or inhibit lipolysis.

In conclusion, this study demonstrated that catecholamine-stimulated lipolysis is enhanced in the presence of glucose, and suggests that glucose is one of the primary substrates for G3P during active lipolysis.

Author contributions NT, KH, XL, and NN conceived and designed research; NT, KH, XL, and NN performed experiments; NT, KH, XL, and NN analyzed data; NT, KH, XL, and NN interpreted results of experiments; NT and KH prepared figures; NT and KH drafted manuscript; NT, KH, XL, and NN edited and revised manuscript; NT, KH, XL, and NN approved final version of manuscript.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval Animal Experimental Committee of University of Shiga Prefecture approved this experimental protocol (Ref #30-10).

Consent to participate Not applicable.

Consent for publication Not applicable.

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