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Original Article

Role of GABA and its receptors in anti-adipogenesis in cultured adipocytes

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Abstract

Health benefits of GABA and GABA-enriched foods via anti-obesity activity have been demonstrated in animals. However, the functional roles of GABA especially as non-neurotransmitter in adipocytes have not been thoroughly clarified. Thus, this study aimed to assess the impact of GABA on adipogenesis and lipolysis using 3T3-L1 adipocytes. Oil Red O staining was used to assess lipid accumulation and glycerol release was determined as an indicator of lipolysis. The results showed that treatment with GABA (100-500 µM) during adipocyte maturation decreased lipid accumulation by ~20-30%. GABA clearly induced the release of glycerol in a dose-dependent manner within 4 h of treatment, and GABA at 500 µM increased glycerol release about two-fold relative to control. GABA-mediated lipolysis depended on both GABA-A and GABA-B receptors as well as on β-adrenergic receptors. The findings suggest that GABA exhibits non-neuronal functions by promoting lipolysis and by suppressing fat accumulation in adipocytes, which potentially support anti-obesity activity of GABA and GABA-enriched products.

Keywords: GABA, GABA receptor, adipogenesis, lipolysis, adipocyte

1. Introduction

Obesity has increased dramatically in the developed countries, and currently is on rise also in developing and underdeveloped economies. According to the World Health Organization, there are 1.9 billion overweight people in the world, of whom more than 650 million are obese (World Health Organization [WHO], 2018). Obesity is a risk factor contributing to major diseases, including type 2 diabetes, hypertension, and atherosclerosis (Walley, Blakemore, & Fro guel, 2006). Adipocytes are lipid buffering sites that store excess plasma triglycerides by lipogenesis, later releasing them during periods of metabolic deficiency by lipolysis via the TAG-fatty acid cycle (Lafontan, 2009; Large *et al.*, 2004). Prolonged positive energy balance caused by excessive caloric intake without commensurate energy consumption leads to obesity (Bergman & Ader, 2000; Guyenet & Schwartz, 2012). Thus, compounds that suppress adipocyte lipogenesis and promote lipolysis have been proposed as anti-obesity agents (Andersen *et al.*, 2010).

White adipose tissue (WAT) appears to have a diffuse sympathetic innervation and expresses β3- adrenergic receptors, which are emerging as important regulators of body fat mass (Bartness & Bamshad, 1998; Cinti, 2005; Dodt *et al.*, 2003; Francois *et al.*, 2018). GABA is a classic inhibitory neurotransmitter mainly exerting its biological effect by suppressing neuronal excitability. Activation of GABA-A receptor-mediated signaling in the lateral hypothalamus suppresses food intake and reduces body weight (Turenius *et al.*, 2009). However, it also has humoral actions outside the CNS, including in pancreas, endothelium, gastrointestinal tract, adrenal medulla, and placenta (Gladkevich *et al.*, 2006; Sen *et al.*, 2016) and possibly affects adipocyte function in a paracrine fashion (Nicolaysen *et al.*, 2007). GABA is found in various foods and beverages (Diana, Quílez, & Rafecas, 2014), which has further prompted studies on the non-neuro-

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nal roles of GABA (Ho *et al.*, 2012; Lim *et al.*, 2016). GABA was thought to play some roles in ameliorating metabolic disorders such as obesity. Both GABA-A and GABA-B receptors are also expressed in adipose tissues (Nakamura *et al.*, 2011; Nicolaysen *et al.*, 2007), but their functional roles in adipocytes are poorly defined.

Thus, the present study aimed to demonstrate the role of GABA on pre-adipocyte differentiation and lipogenesis and to investigate the participation of GABA-A or GABA-B receptors. The inhibitory actions of GABA found in this study may provide a potential treatment for obesity by targeting GABA paracrine signaling including utilizing this amino acid in the diet.

2. Materials and Methods

2.1 Chemicals

3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, Oli Red O, isopropanol (Isop), formalin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide), GABA, baclofen (GABA-B agonist), bicuculline (GA BA-A antagonists), CGP52432 (GABA-B antagonists), propranolol hydrochloride (β-adrenergic receptor antagonist) and adipolysis assay kit were purchased from Sigma-Aldrich, St. Louis, MO. Dulbecco modified Eagle medium (DMEM) with either calf bovine serum (CBS), or fetal bovine serum (FBS), trypsin/EDTA, and penicillin/streptomycin were purchased from GIBCO, Grand Island, NY.

2.2 3T3-L1 pre-adipocyte culture and differentiation

Mouse embryo 3T3-L1 cell line was obtained from the American Type Culture Collection (ATCC, CL-173). 3T3-L1 pre-adipocytes were grown in normal medium (high [25 mM] glucose-DMEM, supplemented with 10% CBS) at 37 °C in 5% CO_2. For subculturing, the medium was removed, and cells were detached with 0.25% trypsin and 0.2 g/L EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS). For cell differentiation, the 3T3-L1 pre-adipocytes were cultured for 2 days in differentiation media (high glucose-DMEM supplemented with 10% FBS, 1 µM dexamethasone, 0.5 mM IBMX, and 10 µg/mL insulin). At the end of day 2, the media were replenished with maintenance media (high glucose-DMEM with 10% FBS and 10 µg/mL insulin). Media were refreshed every 2 days until day 10.

2.3 Cell viability assay

Cell viability was measured using the MTT assay. 3T3-L1 pre-adipocyte cells in 96-well plates were exposed to GABA (200 - 1000 µM) during adipocyte differentiation. Two hours before the end of the GABA treatment, MTT reagent was added to each well (MTT final concentration 50 µg/mL). The formazan crystal produced in viable cells was dissolved in 200 µL of 100% isopropanol and the absorbance at 595 nm was read using a microplate reader. The viability was calculated as percentage of control or untreated cells.

2.4 Lipid accumulation by Oil Red O staining

3T3-L1 cells were treated with GABA (50 - 500 µM) for 10 days during the differentiation period. Cells were washed twice with ice-cold PBS, fixed with 10% v/v formalin for 1 h, rinsed with 60 % isopropanol, and stained with 3.5 mg/mL Oil Red O solution for 10 min. The stained cells were rinsed three times with PBS. Stained oil droplets in 3T3-L1 cells were extracted with 100% isopropanol and the absorbance was mea-sured at 570 nm using a microplate reader.

2.5 Lipolysis assay by glycerol production

Fully differentiated adipocytes were fasted overnight in serum-free medium and then treated with GABA (250-1000 µM) in Hanks' buffer for 4 h. The medium was then collected to measure the glycerol using the adipolysis assay kit, which used a coupled enzyme assay involving glycerol kinase and glycerol phosphate oxidase, and the product was measured colorimetrically with a spectrophotometer (570 nm). Isoproterenol (Isop) was used as a positive control for lipolysis.

2.6 Statistical analysis

All data are presented as mean ± standard error of the mean, of at least three separate experiments. The data were subjected to analysis of variance (ANOVA) followed by LSD tests. P values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1 GABA reduced lipid accumulation during lipogenesis

GABA at high concentrations (200-1000 µM) showed no indication of toxicity as judged by the MTT assay (Figure 1B). During 10 days of maturation of adipocytes, TAG accumulation by the mature 3T3-L1 cells was approximately 3 times higher than by pre-adipocytes, as indicated by Oil Red O staining (Figure 1C). 3T3-L1 cells grown in the presence of GABA (100 µM and above) for the entire maturation period had a modestly reduced lipid content (by ~30%) (Figure 1D).

According to the discovery that GABA receptors and GABA-synthesizing enzyme L-glutamic acid decarboxylase (GAD) were expressed in adipocytes (Nakamura *et al.*, 2011; Nicolaysen *et al.*, 2007), a non-neuronal role of GABA was possible due to the paracrine mechanism: GABA released from one adipocyte triggered the receptors on neighboring cells (Tian *et al.*, 2011). The potential of GABA as anti-obesity agent was previously demonstrated in mice fed with a high fat diet, showing that oral treatment with GABA (in drinking water) reduced both adipocyte size and epidermal fat mass (Tian *et al.*, 2011). Up to now, there is still only a limited number of studies exploring the anti-obesity mechanisms of GABA. The present study demonstrates a direct effect of GABA on lipid accumulation in cultured adipocytes. GABA-mediated down-regulation of lipogenic genes was thought to be a potential mechanism, since various GABA-enriched extracts were demonstrated to suppress the expression of adipogenesis related genes, including transcription factors (C/ EBP-β, C/EBP-α, SREBP-1c, and PPAR-γ) and lipogenesis enzymes (aP2, LPL, and FAS) in 3T3-L1 cells and animals (Ho *et al.*, 2012; Lim *et al.*, 2016).

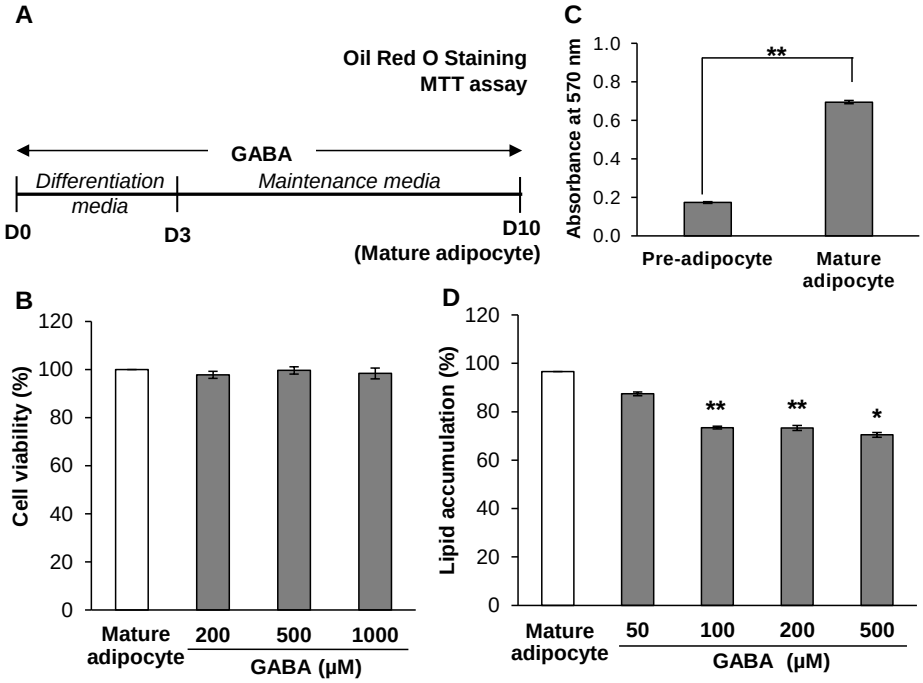

Figure 1. Effects of GABA on cell viability and lipid accumulation of 3T3-L1 adipocytes. (A) Schematic of the protocol. During the entire 10-day period, cells were exposed to high glucose + insulin and then harvested at day 10 for analyses. (B) The effects of GABA on viability of adipocytes as observed with an MTT assay. (C, D) Lipid accumulation quantified by Oil Red O staining with absorbance measured at 570 nm. The results on y-axes are expressed in proportion to the untreated adipocytes in the same experiment. The data are mean values ± SEM (n = 3-7) $*p < 0.05$, $**p < 0.01$.

3.2 GABA stimulated lipolysis

Isoproterenol (10 μM) increased glycerol production rate 2.3-fold, suggesting increased TAG hydrolysis after only 4 h. Isoproterenol is a non-selective β-adrenergic receptor agonist that has been widely used as a standard compound to induce lipolysis (Louis *et al.*, 2014; Muller *et al.*, 2003). GABA also increased glycerol production in a dose-related manner (Figure 2B). This suggests that GABA stimulated triglyceride hydrolysis from adipocytes, as shown by the release of glycerol into cultured medium. According to this measurement, the release of glycerol was observed shortly after the treatment (4 h) and continuously increased, so that a sample collected at 24 h was beyond the measurement range.

As regards lipid accumulation, no changes in lipid content were observed at 4 h of treatment by Oil Red O staining (data not shown), so 24 h treatments with GABA and isoproterenol were performed. Isoproterenol had a slight depressant action (by 11%) and GABA also reduced lipid accumulation by up to 27% (Figure 2C). The effects of either isoproterenol or GABA on lipolysis were far greater in magnitude and over a shorter time than on lipogenesis. Thus these actions on lipolysis are probably functionally consequential and this accords with the known β3-adrenoceptor action (Holm, 2003).

Generally, adipose lipolysis is an important process that controls free fatty acid (FFA) concentration in the blood. It is considered an anti-obesity mechanism that reduces adipose tissue mass. It should be noted that FFA liberated into the bloodstream due to endoplasmic reticulum stress contributed to lipotoxicity by inducing impaired insulin sensitivity of adipocytes (Deng *et al.*, 2012). GABA-induced lipolysis should be considered for future use although GABA treatment showed improved insulin sensitivity in mice fed with a high fat diet (Tian *et al.*, 2011).

3.3 The roles of GABA-A and GABA-B receptors

Fragmentary evidence suggests that adipocytes express both GABA-A and GABA-B receptors (Nakamura *et al.*, 2011; Nicolaysen *et al.*, 2007). We therefore used receptor subtype ligands to determine which of these was mediating the action of GABA.

Baclofen, a GABA-B receptor agonist, also stimulated lipolysis similar to GABA (Figure 3A) suggesting largely GABA-B mediated responses. We further investigated receptor subtype involvement using selective antagonists, bicuculline (GABA-A) and CGP52432 (GABA-B). Both antagonists reduced GABA-mediated lipolysis (Figure 3B). However, together they depressed lipolysis to below the baseline both with and without GABA.

The roles of GABA (Tian *et al.*, 2011) and GABA-enriched germinated brown rice (Ho *et al.*, 2012; Lim *et al.*, 2016) were previously demonstrated in animal models. When mice with high fat diet induced obesity received germinated brown rice, their adipose tissues showed decreased expression of several adipogenesis enzymes, such as fatty acid synthase (Cinti), and increased expression of certain lipases such as hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Ho *et al.*, 2012). Currently, the signaling path-

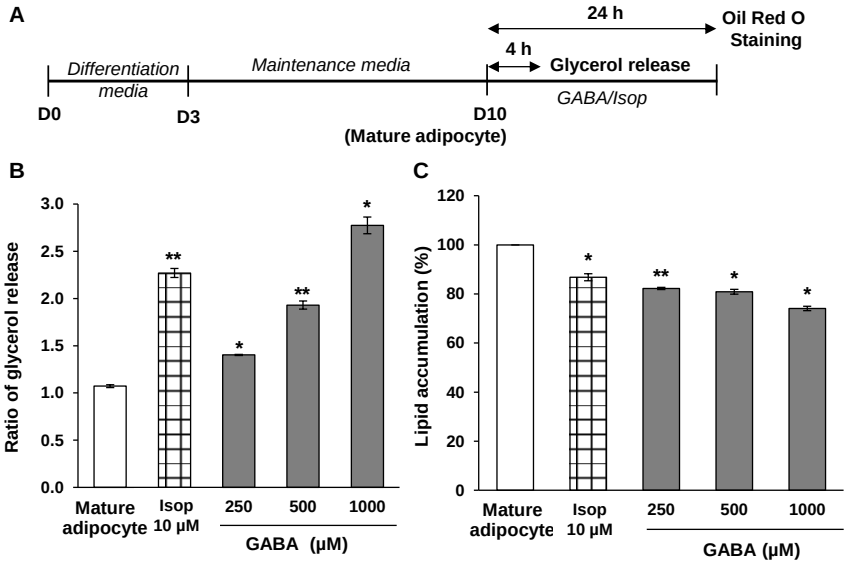

Figure 2. Effects of GABA on adipolysis. (A) Schematic of the protocol. (B) The effects of isoproterenol (Isop), or GABA on lipolysis in 3T3-L1 mature adipocytes, measured as glycerol release after 4 h. (C) Intracellular lipid accumulation using Oil Red O staining assay. 10 µM isoproterenol was used as a positive control of lipolysis. Results are expressed in proportion to "mature adipocytes". Values are mean ± SEM (n = 3-7). $^{*}p < 0.05$, $^{**}p < 0.01$

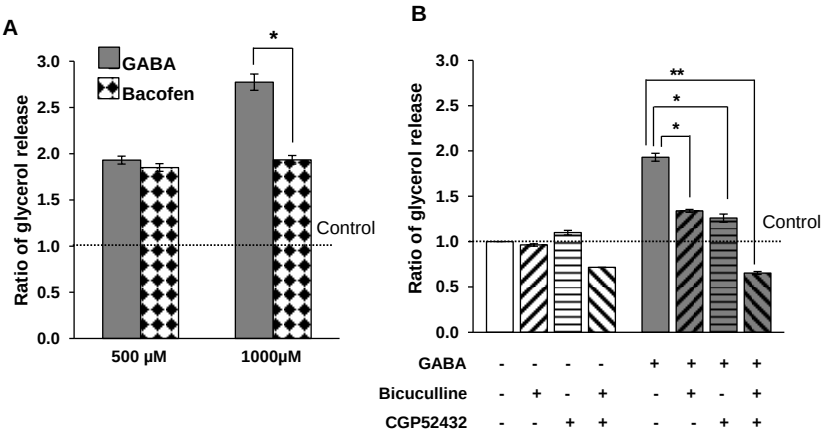

Figure 3. GABA receptor subtype and lipolysis. The release of glycerol from 3T3-L1 mature adipocytes was determined after 4 h of treating cells with (A) GABA or baclofen (GABA-B receptor agonist) or (B) with 500 µM GABA in the absence or presence of 250 µM bicuculline (GABA-A receptor antagonist), or of 1 µM CGP52432 (GABA-B receptor antagonist). All tested drugs were added for "4 h" as in Figure 2A. The data are mean values ± SEM (n = 3-7). $^{*}p < 0.05$, $^{**}p < 0.01$

ways coupling adipocyte GABA receptor-activation to fat metabolism are unclear. However, while it is too early to draw a conclusion, the up/down-regulation of certain regulatory proteins is possibly involved in GABA-mediated adipocyte fat metabolism.

3.4 β-adrenergic receptor antagonist inhibits GABA-mediated adipocyte lipolysis

In addition to GABA receptor antagonists, the present study tested the effects of β-adrenergic receptor antagonist, propranolol. This β-blocker should inhibit the action of isoproterenol but not of GABA. However, actually the propranolol blocked the lipolysis equally well, whether induced by isoproterenol or GABA (Figure 4). This suggests common signaling pathways for isoproterenol and GABA.

From the current study, the receptor ligands assessed were acting specifically on their respective putative targets suggesting the relationships between GABA and β-adrenergic receptors on fat metabolism of adipocytes. Discussion of the link between GABAergic and adrenergic receptors is not possible here, because their non-neuronal roles in adipocytes have not been reported elsewhere. However, the present results provide early evidence demonstrating the relationships of these two receptors in non-neuronal tissues.

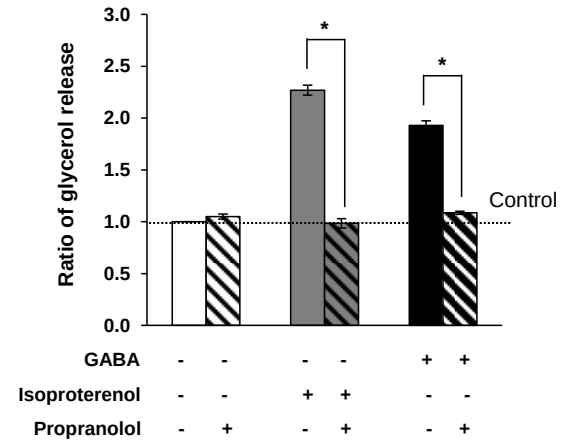

Figure 4. Role of β-adrenergic receptor on GABA-induced lipolysis. The release of glycerol from 3T3-L1 mature adipocytes was determined after 4 h of treating cells with 500 μM GABA or 10 μM isoproterenol in the presence or absence of 1 μM propranolol (β-adrenergic receptor agonist). All the tested drugs were added for "4 h" as in Figure 2A. The data are mean values ± SEM (n = 3-7). $^{*}p < 0.05$.

4. Conclusions

This study provided evidence that GABA plays some roles in fat metabolism of adipocytes by reducing adipogenesis/lipid accumulation and by promoting lipolysis in adipocytes. Both GABA-A and GABA-B receptors as well as β-adrenergic receptors are responsible for GABA-mediated lipolysis. This finding also provides a possible mechanism to support the anti-obesity health benefits of GABA and GABA-enriched products.

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