Folic Acid Supplementation Modifies β-Adrenoceptor–Mediated *In Vitro* Lipolysis of Obese/Diabetic (+*db*/+*db*) Mice

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The effects of folic acid (5.7 and 71 μg/kg, 4 weeks) consumption on the β-adrenoceptors (β-ARs)–elicited lipolysis *in vitro* of the abdominal adipocytes of lean/control (+*m*/+*db*) and obese/diabetic (+*db*/+*db*) mice (female) were investigated. β-AR agonists (salbutamol, a β_2-AR agonist; BRL 37344 and CGP 12177, β_3-AR agonists; adrenaline, a β-AR agonist)–mediated lipolysis, β_2-, and β_3-ARs protein expression of the adipose tissues after folic acid consumption were evaluated. Our results demonstrate that a smaller magnitude of the basal (spontaneous) and the β-AR agonists–triggered lipolysis was observed in +*db*/+*db* mice, and folic acid supplementation (71 μg/kg) resulted in an improvement of both the baseline and the β-ARs–mediated lipolysis. In controls, a lower β_2- and β_3-ARs protein expression of the adipose tissues was detected in +*db*/+*db* mice, compared to +*m*/+*db* mice. In both strains fed with folic acid (71 μg/kg), a reduction of β_2-AR protein expression was observed compared to the respective controls. In +*db*/+*db* mice, folic acid (5.7 and 71 μg/kg) consumption caused a dose-dependent increase of β_3-AR protein expression compared to controls. We demonstrate that lipolysis elicited by β-AR (β_2- and β_3-ARs) agonists was blunted in +*db*/+*db* mice. Folic acid consumption has significant modulatory effects on β-ARs protein expression and lipolysis. Exp Biol Med 234:1047–1055, 2009

Key words: β-adrenoceptor; lipolysis; folic acid; adipocytes; +*db*/+*db* mice

Introduction

Obesity is a risk factor for the development of metabolic diseases, especially type 2 diabetes mellitus (T2DM), which are major health problems of the population in modern society (1). Obesity results from a reduced lipolytic activity of adipose tissue (1, 2). β-Adrenoceptors (β-ARs) (mainly β_3-AR), which are widely expressed in adipose tissues (3–5), play a crucial role in lipolysis. Stimulation of β-ARs induces phosphorylation of the hormone-sensitive lipase and triggers lipolysis through the cAMP-PKA–dependent signaling pathway (3). Interestingly, activation of β-ARs improves obesity and metabolic disorders in rodents (6, 7) and humans (4, 8).

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Folic acid is a water-soluble B vitamin abundant in vegetables, fruits, grain, and dairy products (9), and the recommended daily intake of folic acid is 400 μg/70 kg for adults (10). Folic acid supplementation (10 mg/day) lowers the plasma homocysteine levels in patients with mild to moderate hyperhomocysteinemia, and improves the flow-mediated endothelium-dependent dilatation (11). The homocysteine-lowering effects of folic acid may reduce the risk of cardiovascular disease development, especially in T2DM (9, 12). In addition, folic acid supplement decreases LDL-oxidation *in vitro* (13), which may slow down the progression of atherosclerosis. Interestingly, folic acid supplementation can produce non–homocysteine-dependent effects (14). Restoration of endothelial nitric oxide synthase (eNOS) reaction in T2DM patients by folate supplementation is independent of homocysteine-lowering effects (15).

Increased body mass index (BMI), percentage of body fat, and absolute amounts of central and peripheral fat depots are associated with a decrease in serum folic acid level (16). However, the modulatory effects of oral folic acid supplementation on lipolysis are rarely studied. Therefore, we investigated whether oral folic acid supplementation could modify β-ARs–mediated lipolysis of the abdominal (omental) adipose tissues (a metabolically active fat depot) (17). Because of the lesser cardio-stimulatory effects of β_2- and β_3-ARs compared to β_1-AR, we thus evaluated β_2-AR and β_3-AR agonists–mediated lipolysis in this study using lean/non-diabetic (*+m/+db*) mice and obese/diabetic (*+db/+db*) mice, an animal model for human obesity and T2DM research (18, 19).

So far, most cardiovascular studies (especially related to diabetes) were performed using male animals. Unfortunately, studies performed using female animal models are relatively scarce, except for the overwhelming efforts concentrated on topics related to oestrogen therapy. In view of the importance of lipolysis in the development of cardiovascular diseases and DM, we therefore hypothesized that oral folic acid supplementation (5.7 and 71 μg/kg, daily for 4 weeks) differentially modifies the *in vitro* lipolysis of the abdominal (omental) adipose tissues elicited by β-ARs (β_2- and β_3-ARs) activation under obese/diabetic conditions.

Materials and Methods

Animals. C57BL/KsJ mice (female; ~6 months old) (non-diabetic/lean (*+m/+db*) mice: 24.5 ± 2.3 g; diabetic/obese (*+db/+db*) mice: 58.2 ± 3.4 g) were housed under a 12:12-hour light-dark cycle (7:00 am, ON; 7:00 pm, OFF) (relative humidity: 55–60%; temperature: 22 ± 1°C) and were given standard chow (folate content: 7.9 ppm) (Prolab® RMH 2500, Australia) and water *ad libitum* before they were sacrificed. Food consumption was measured and recorded every day. The Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (HKSAR, PR China) approved all experiments performed in this study (approval no. 04/054/MIS and 07/003/MIS). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and to limit the number of animals used in these experiments.

Drug Administration and Body Weight Determination. Folic acid was dissolved in Nanopure deionized water and the stock solution was stored at –20°C. The working solution for oral gavage was freshly prepared daily from the stock. Mice were divided randomly into three groups (control, folic acid (5.7 μg/kg, equivalent to 400 μg for a 70-kg adult), and folic acid (71 μg/kg, equivalent to 5 mg for a 70-kg adult)) ($n = 4$–5 for each group) (doses of folic acid used in rodents previously) (14). Drugs (or distilled water, which served as control) were administered orally, using a feeding needle, daily for 4 consecutive weeks before the animals were sacrificed for experiments. It is important to point out that, at the beginning of this study, we were not sure whether folic acid administration altered the daily chow consumption by mice. It was therefore decided to administer folic acid to mice by oral gavage (so as to be certain about the dose of folic acid received by each individual mouse) rather than adding folic acid into the chow/water. A change in body weight and chow consumption of each individual mouse during the course of folic acid administration was monitored and recorded daily.

Determination of Plasma Homocysteine and Folate Levels. Blood samples were drawn from the tail vein weekly, promptly centrifuged, and stored at –80°C. Plasma (total) homocysteine (referring to the combination of free reduced homocysteine, mixed disulfides, and protein-bound homocysteine) and folate levels were measured using high performance liquid chromatography (HPLC) with fluorescence detection and commercial assay (Beckman), respectively, as described elsewhere (20–22). All samples were measured in triplicate.

Isolation of Adipocytes and Lipolysis Experiment. The abdomen of each mouse was opened, and the abdominal (omental) adipose tissue (a metabolically active fat depot) (17) was harvested. The amount of adipose tissue collected from a *+m/+db* mouse was ~0.4–0.7 g ($n = 50$), whereas ~4.2–6.0 g ($n = 50$) was collected from a *+db/+db* mouse.

Adipocytes were prepared as previously reported by Rodbell (23), with minor modifications. Harvested abdominal (omental) fat pads were rinsed 3–4 times to remove the blood in Krebs'-Ringer solution of the following composition (m*M*): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, and $CaCl_2$ 1.8 (gassed with 16% O_2/5% CO_2/79% N_2 (pO_2 ~100 mm Hg); pH 7.4; 37 ± 1°C). Washed adipose tissue was minced using a pair of scissors and incubated in Krebs'-Ringer solution containing collagenase (0.5 mg/ml) (supplemented with 3% bovine serum albumin (BSA)) (Sigma-Aldrich, USA) at 37°C in a shaking water-bath for 60 mins. After the enzymatic

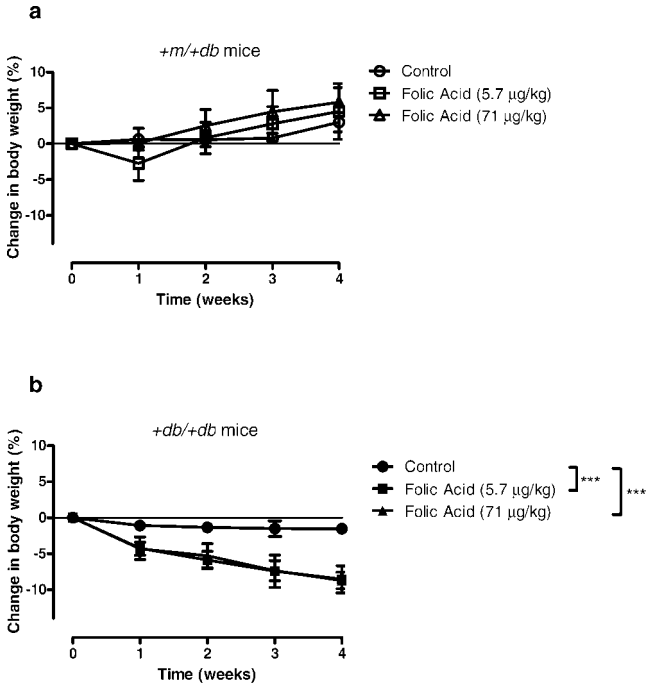

Figure 1. Percentage (%) change in body weight of +*m*/+*db* mice (open symbols) and +*db*/+*db* mice (closed symbols) orally fed with folic acid (5.7 µg/kg (□, ■) and 71 µg/kg (△, ▲)), compared to the respective drug-free controls (+*m*/+*db* mice (○); +*db*/+*db* mice (●)) (n = 24–25 for each group of mice). Results are expressed as mean ± SEM. *** $P < 0.001$ compared to the controls (two-way ANOVA).

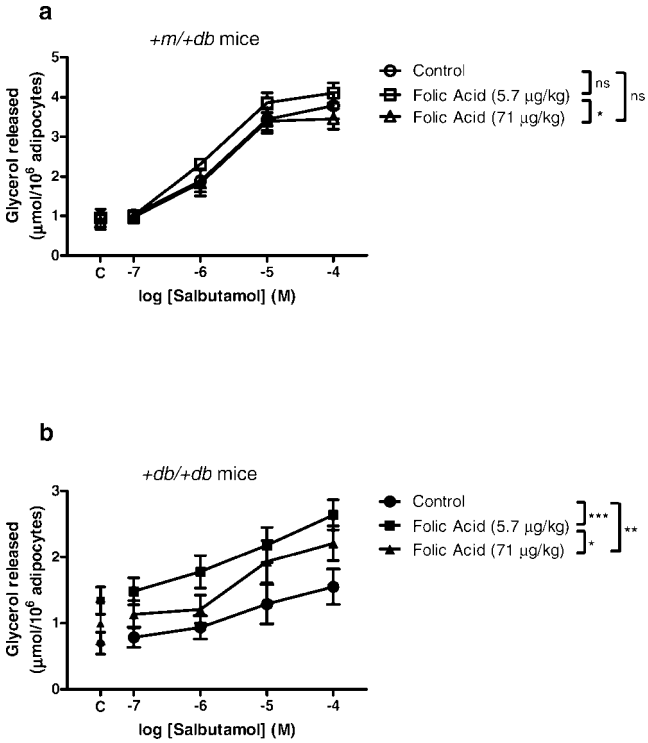

Figure 2. Concentration-response curves for the stimulation of glycerol release, as a measure of the lipolytic response, from the abdominal (omental) adipocytes elicited by β_2-adrenoceptor agonist salbutamol, of +*m*/+*db* mice (a) (open symbols) and +*db*/+*db* mice (b) (closed symbols) orally fed with folic acid (5.7 µg/kg (□, ■) and 71 µg/kg (△, ▲)), compared to the respective drug-free controls (+*m*/+*db* mice (○); +*db*/+*db* mice (●)) (n = 4 for each group of mice). Results are expressed as means ± SEM. *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the respective controls (two-way ANOVA). C, control (drug-free) conditions; ns, not significantly different.

digestion procedures, the adipocytes were filtered through a nylon mesh (180 µm) to remove the connective tissue. Isolated adipocytes collected were washed 3 times with collagenase-free Krebs'-Ringer solution. The number of adipocytes was counted under a phase-contrast microscope (Nikon, Japan) after Trypan Blue staining (to determine the viability/yield of adipocytes harvested) using a hematocytometer. Adipocytes collected were incubated in Krebs'-Ringer solution (supplemented with 3% BSA) and were divided into aliquots and incubated in a final volume of 200 µl (in an Eppendorff tube) at 37°C in a water-bath for 150 mins with graded concentrations of individual lipolytic agent added. After incubation, the lipolysis reaction was terminated by immersing the tubes in an ice-water bath. The amount of glycerol released was measured using the commercially available free glycerol determination kit (Sigma-Aldrich, USA) according to the manufacturer's protocols. The amount of glycerol released (expressed as µg glycerol released/1 × 10^6 adipocytes) was used as an index of lipolysis. All samples were measured in triplicate.

Western Immunoblotting Analysis. Abdominal (omental) adipose tissues were isolated from +*m*/+*db* and +*db*/+*db* mice, as described in previous sections. Tissues were rinsed several times with ice-cold Krebs'-Ringer solution to remove blood before subjecting them to molecular biology experiments. Then, adipose tissues were homogenized in the presence of protease inhibitors to obtain extracts of proteins. Protein concentrations were determined using BCA™ protein assay kit (Pierce, USA). Samples (25 µg of protein per lane) were loaded onto a 10% SDS-polyacrylamide electrophoresis gel. After electrophoresis (90 V, 90 mins), the separated proteins were transferred (12 mA, 60 mins) to polyvinylidene difluoride membrane (PerkinElmer™ Life Sciences, USA). Nonspecific sites were blocked with 5% nonfat dry milk for 120 mins, and the blots were then incubated with anti–β_2-AR antibody, 1:1,000, and anti–β_3-AR antibody, 1:1,000 (Santa Cruz Biotechnology, USA), overnight at 4°C. Anti-rabbit HRP conjugated IgG, 1:1,000 (DakoCytomation, Denmark), was used to detect the binding of its correspondent antibody. Membranes were stripped and re-blotted with anti–β-actin antibody, 1:5,000 (Sigma-Aldrich, USA), to verify an equal loading of protein in each lane. All samples were measured in triplicate. The protein expression was detected with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer™ Life Sciences, USA) and quantified using NIH Image analysis programme.

Chemicals. Physiological salts (GR grade) for preparing Krebs'-Ringer solution were obtained from Merck (Darmstadt, Germany). Adrenaline hydrochloride, forskolin, salbutamol, yohimbine hydrochloride, and folic acid were

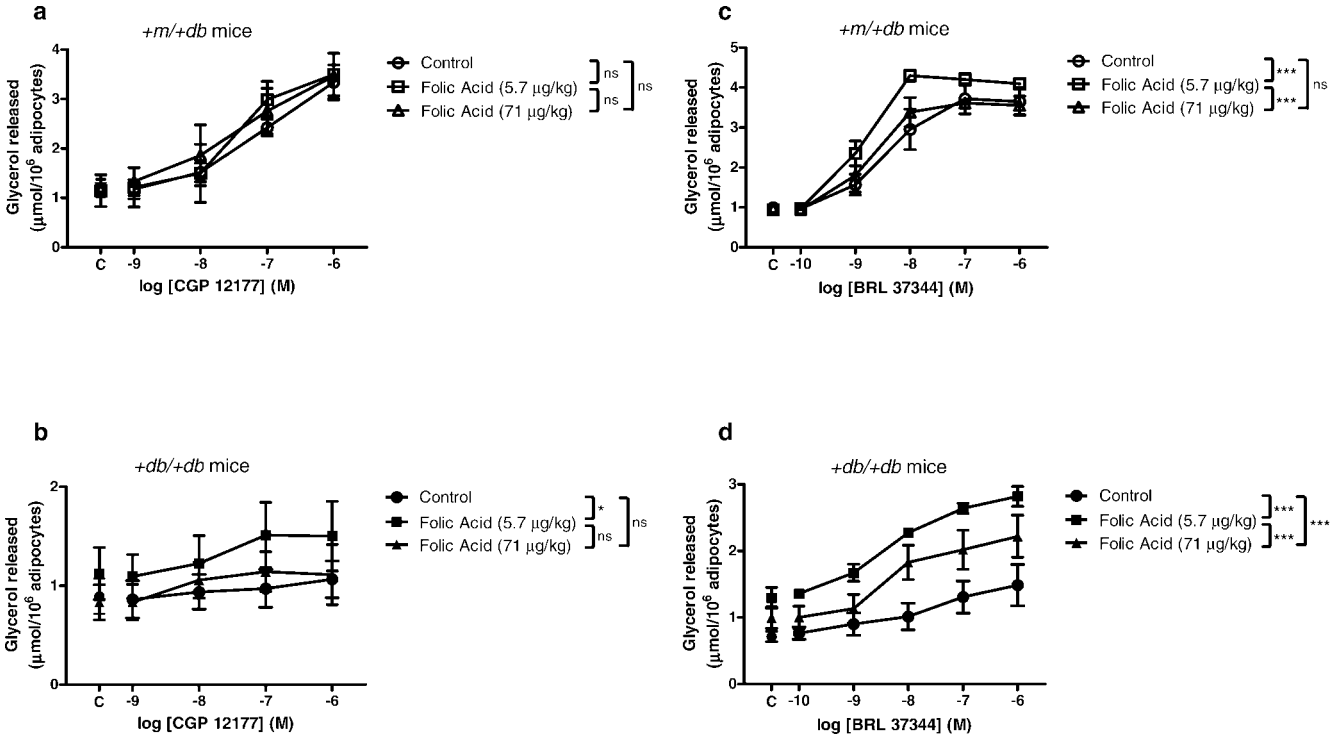

Figure 3. Concentration-response curves for the stimulation of glycerol release, as a measure of the lipolytic response, from the abdominal (omental) adipocytes elicited by CGP 12177 (a, b) and BRL 37344 (c, d) of *+m/+db* mice (open symbols) and *+db/+db* mice (closed symbols) orally fed with folic acid (5.7 µg/kg (□, ■) and 71 µg/kg (△, ▲)), compared to the respective drug-free controls (*+m/+db* mice (○); *+db/+db* mice (●)) ($n = 4$ for each group of mice). Results are expressed as means ± SEM. * $P < 0.05$ and *** $P < 0.001$ compared to the respective controls (two-way ANOVA). C, control (drug-free) conditions; ns, not significantly different.

purchased from Sigma-Aldrich Co. (USA). 8-Bromo-cAMP, BRL 37344 ((R*,R*)-(±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid, sodium salt), ICI 118551 ((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-di hydro-2H-benzimidazol-2-one hydrochloride), SR 59230A (1-(2-ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride) were obtained from Tocris Biosciences (UK).

Statistical Analysis. Data are expressed as means ± SEM; *n* refers to number of mice from which adipose tissues were taken for experiments. Statistical comparisons were performed using Student's *t* test or analysis of variance (one-way or two-way ANOVA) for multiple comparisons, where appropriate. Statistical difference was considered to be significant at a value of $P < 0.05$.

Results

Determination of the Changes in Body Weight and Daily Chow Consumption. There was no change in body weight of *+m/+db* mice after oral folic acid supplementation (5.7 µg/kg and 71 µg/kg) ($n = 24$–25 for each group, $P > 0.05$) (Fig. 1a). In contrast, the body weight of *+db/+db* mice decreased by ~9% after 4-week treatment with folic acid ($n = 24$–25 for each group, $P <$ 0.001, compared to *+m/+db* mice) (Fig. 1b). Despite a decrease in body weight, the daily chow consumption of individual mice remained unchanged (~1.12 g/day in both strains).

Lipolytic Effects of β-Adrenoceptor Agonists. The basal lipolytic activity of adipocytes of *+m/+db* mice was higher than that of *+db/+db* mice (Fig. 2). The effects of different β-AR agonists on lipolysis were studied. Salbutamol (a β_2-AR agonist), CGP 12177, and BRL 37344 (β_3-AR agonists) stimulated lipolysis in both *+m/+db* and *+db/+db* mice in a concentration-dependent manner (Figs. 2 and 3). However, the lipolytic effects of all of the above β-AR agonists (over the same concentration range) in *+db/+db* mice were less than those in *+m/+db* mice (Figs. 2 and 3). The salbutamol-induced lipolysis was inhibited by ICI 118,551 (0.1 and 1 µ*M*, a selective ($_2$-AR antagonist) ($n = 4$ for each group), while the CGP 12177– and BRL 37344–induced lipolytic effect was inhibited by SR 59230A (0.1 and 1 µ*M*, a potent/selective ($_3$-AR antagonist) ($n = 5$ for each group) (data not shown).

The effects of folic acid on the β-AR agonists–induced lipolysis were also studied. Folic acid (5.7 µg/kg and 71 µg/kg) itself did not significantly affect the basal lipolytic activity in both *+m/+db* and *+db/+db* mice. Moreover, folic acid (5.7 µg/kg and 71 µg/kg) supplementation did not alter the salbutamol-induced lipolytic responses in *+m/+db* mice (Fig. 2). However, folic acid (5.7 µg/kg) significantly

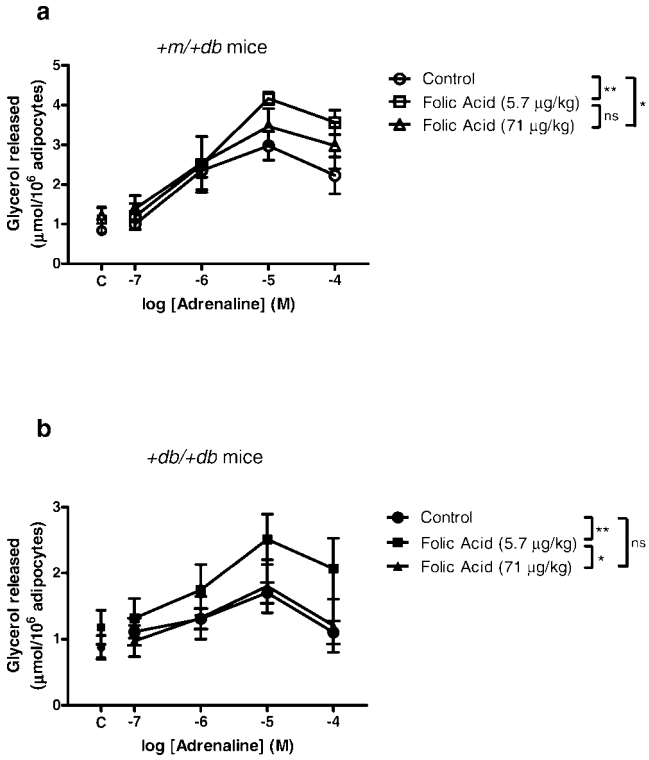

Figure 4. Concentration-response curves for the stimulation of glycerol release, as a measure of the lipolytic response, from the abdominal (omental) adipocytes elicited by adrenaline, of +*m*/+*db* mice (open symbols) and +*db*/+*db* mice (closed symbols) orally fed with folic acid (5.7 µg/kg (□, ■) and 71 µg/kg (△, ▲)), compared to the respective drug-free controls (+*m*/+*db* mice (○); +*db*/+*db* mice (●)) ($n = 4$ for each group of mice). Results are expressed as means ± SEM. * $P < 0.05$ and ** $P < 0.01$ compared to the respective controls (two-way ANOVA). C, control (drug-free) condition; ns, not significantly different.

enhanced the lipolytic effects of salbutamol in +*db*/+*db* mice. There was a paradoxical decrease in salbutamol-induced lipolytic effect when a high dose of folic acid (71 µg/kg) was used in +*db*/+*db* mice.

In general, folic acid supplementation (5.7 µg/kg and 71 µg/kg) did not affect the CGP 12177– and BRL 37344–induced lipolysis in +*m*/+*db* mice (Fig. 3a and 3c), although a slight enhancing effect caused by a low dose of folic acid (5.7 µg/kg) of a BRL 37344–mediated response was noticed. On the other hand, in +*db*/+*db* mice, folic acid (5.7 µg/kg) significantly potentiated the CGP 12177– and BRL 37344–induced lipolysis (Fig. 3b and 3d). Similar to salbutamol, a high dose of folic acid (71 µg/kg) caused a paradoxical decrease in the CGP 12177– and BRL 37344–induced lipolysis.

Experiments were extended to study the effects of adrenaline, which is an endogenous β-AR agonist, in lipolysis (Fig. 4). Adrenaline induced lipolysis in both +*m*/+*db* mice and +*db*/+*db* mice, but its effect on the former species was greater. The lipolytic profile of adrenaline was slightly different from that of all other synthetic β-AR agonists we tested above. Adrenaline showed a maximal lipolytic action at 10 µ*M*. However, a higher concentration of adrenaline (i.e. 100 µ*M*) led to a decrease in lipolysis, and such a "suppressed phase" of lipolysis could be reversed by yohimbine (10 µ*M*, an α_2-adrenoceptor antagonist) ($n = 4$ for each group) (data not shown). A low dose of folic acid (5.7 µg/kg) potentiated the adrenaline-induced lipolysis, but this potentiation effect was not obvious with a high dose of folic acid (71 µg/kg) (Fig. 4).

Effects of Folic Acid on cAMP-PKA Activators–Induced Lipolysis. Similar to all β-AR agonists examined above, forskolin (an adenylate cyclase activator) and 8-bromo-cAMP (8-Br-cAMP) (a cell-permeable cAMP analogue which stimulates PKA) induced lipolysis in both +*m*/+*db* and +*db*/+*db* mice (Fig. 5). Folic acid (5.7 µg/kg and 71 µg/kg) failed to alter forskolin- and 8-Br-cAMP–induced lipolysis in +*m*/+*db* mice. The forskolin- and 8-Br-cAMP–induced lipolysis in +*db*/+*db* mice was potentiated by the low dose of folic acid (5.7 µg/kg). However, this potentiation was not obvious when a high dose of folic acid (71 µg/kg) was used.

Protein Expression of β-Adrenoceptors. The protein expressions of β_2- and β_3-ARs in the adipose tissues of +*db*/+*db* mice were significantly lower than those of +*m*/+*db* mice (Fig. 6). The protein expressions of β_2-AR in both +*m*/+*db* and +*db*/+*db* mice were decreased by the high dose (71 µg/kg), but not by the low dose, of folic acid (5.7 µg/kg) treatment. In contrast, the protein expression of β_3-AR in +*m*/+*db* mice was not altered by folic acid (5.7 and 71 µg/kg) (Fig. 6a and 6b), whereas the protein expression of β_3-AR in +*db*/+*db* mice was significantly increased by the low dose of folic acid (5.7 µg/kg) (Fig. 6c and 6d). The high dose of folic acid (71 µg/kg) did not cause a further increase of the protein expression of β_3-AR in +*db*/+*db* mice (Fig. 6c and 6d).

Effects of Folic Acid Consumption on the Plasma Level of Folate and Homocysteine. A lower plasma folate level was detected in control (drug-free) +*db*/+*db* mice (29.62 ± 4.08 µg/l) compared to +*m*/+*db* mice (45.22 ± 6.13 µg/l) ($P < 0.01$). The plasma levels of folate of mice were elevated after folic acid consumption (+*m*/+*db* mice, folic acid (5.7 µg/kg): 98.73 ± 5.07 µg/l, folic acid (71 µg/kg): 181.46 ± 7.12 µg/l) (+*db*/+*db* mice, folic acid (5.7 µg/kg): 56.32 ± 6.03 µg/l, folic acid (71 µg/kg): 122.37 ± 9.54 µg/l). To investigate if the effects of folic acid on β-ARs–mediated lipolysis were due to its possible homocysteine-lowering effects, the plasma level of homocysteine was measured. The plasma level of homocysteine in +*db*/+*db* mice was 3.5-fold higher than in +*m*/+*db* mice (25.6 ± 2.1 µ*M* versus 7.3 ± 1.3 µ*M*; $P < 0.01$). However, the plasma homocysteine levels in both strains remained unchanged after the folic acid treatments (Table 1).

Discussion

In the present study, the effects of folic acid on β-ARs–mediated lipolysis were investigated in an animal model of

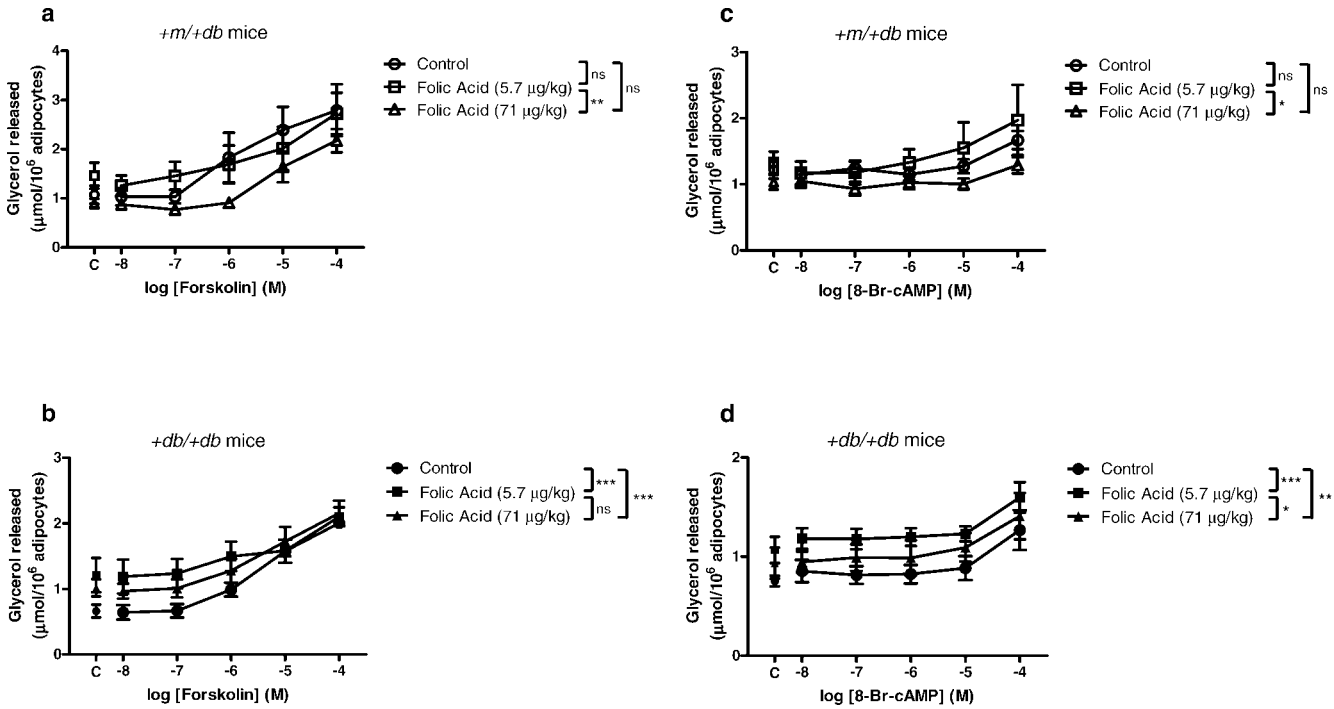

Figure 5. Concentration-response curves for the stimulation of glycerol release, as a measure of the lipolytic response, from the abdominal (omental) adipocytes elicited by forskolin (a, b) and 8-bromo-cAMP (8-Br-cAMP) (c, d) of +*m*/+*db* mice (open symbols) and +*db*/+*db* mice (closed symbols) orally fed with folic acid (5.7 μg/kg (□, ■) and 71 μg/kg (△, ▲)), compared to the respective drug-free controls (+*m*/+*db* mice (○); +*db*/+*db* mice (●)) ($n = 4$ for each group of mice). Results are expressed as means ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the respective controls (two-way ANOVA). C, control (drug-free) condition; ns, not significantly different.

diabetes. Different types of diabetic animal models (6, 8, 24) have been used to evaluate the effects of β-ARs activation on the lipolysis responses of epididymal (24, 25), subcutaneous (8, 26), and retroperitoneal fat depots (24). However, inconsistent results were yielded. It is important to point out that, unlike the abdominal (omental) adipose tissues used in our study, the fat depots employed in the studies mentioned above are not metabolically active. Therefore, they may not contribute significantly to the erratic lipid profiles observed in diabetes mellitus/obesity.

Effects of β_1-AR activation by various β_1-AR agonists on lipolysis have been studied extensively in both rodents and humans. However, most β_1-AR agonists studied suffered from the fact that β_1-AR activation leads to cardiac stimulation, which can be detrimental, especially in diabetic patients with cardiovascular problems. Instead, in this study, we concentrated our efforts and evaluated β_2-AR and β_3-AR activation (both β_2-AR and β_3-AR are found in adipose tissues), which have a lesser degree of cardio-stimulatory responses. Our study showed that the lipolysis response in +*db*/+*db* mice was blunted, which was probably associated with a decreased activity/activation of β_2- and β_3-ARs. This hypothesis is supported by the observations that the lipolytic effects caused by salbutamol (a β_2-AR agonist), CGP 12177 and BRL 37344 (β_3-AR agonists), and adrenaline (a β_2- and β_3-AR agonist) in +*db*/+*db* mice were less than those observed in +*m*/+*db* mice. An interesting finding observed in our study was that adrenaline elicited a "sub-maximal" lipolytic response in both +*m*/+*db* and +*db*/+*db* mice. The lipolytic effect of adrenaline was reduced at 100 μM, and

Table 1. Effects of Oral Folic Acid Supplement (5.7 and 71 μg/kg) on the Plasma (Total) Homocysteine Levels of +*m*/+*db* and +*db*/+*db* Mice

	+*m*/+*db* mice			+*db*/+*db* mice		
Homocysteine (μM)	Control	Folic acid (5.7 μg/kg)	Folic acid (71 μg/kg)	Control	Folic acid (5.7 μg/kg)	Folic acid (71 μg/kg)
1st week	7.1 ± 1.3	6.9 ± 1.2	7.3 ± 1.3	25.6 ± 2.1*	25.4 ± 1.6	27.3 ± 1.7
2nd week	7.2 ± 1.1	7.3 ± 0.7	7.1 ± 1.1	26.1 ± 1.7*	25.9 ± 2.0	25.2 ± 1.5
3rd week	6.9 ± 1.2	7.0 ± 1.3	6.9 ± 1.3	24.8 ± 1.5*	24.4 ± 1.8	26.1 ± 1.9
4th week	7.0 ± 1.0	7.4 ± 0.4	7.2 ± 1.3	25.2 ± 1.5*	26.3 ± 1.7	25.8 ± 1.2

Data are expressed as means ± SEM. * $P < 0.01$ (unpaired Student's *t* test) (+*m*/+*db* mice (control) versus +*db*/+*db* mice (control)) ($n = 24$–25).

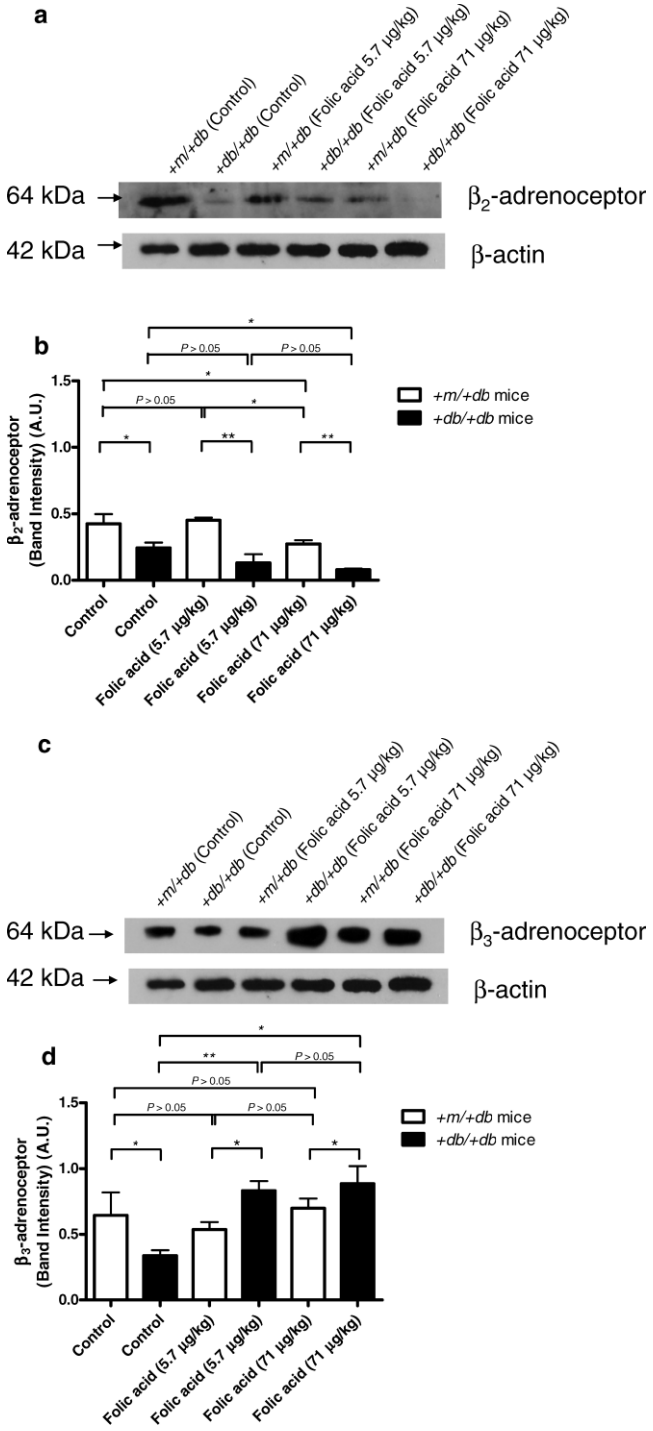

Figure 6. A comparison of the effects of oral folic acid supplementation (4 weeks, 5.7 and 71 μg/kg) on protein expressions of β_2-adrenoceptor (a, b), and β_3-adrenoceptor (c, d) of the abdominal (omental) adipose tissues of *+m/+db* and *+db/+db* mice. β-actin was measured as a loading control for normalization of the protein band intensity. Results are expressed as mean (arbitrary units) ± SEM (normalized to β-actin content) of four independent experiments. * $P < 0.05$ and ** $P < 0.01$ (two-way ANOVA).

this phenomenon was not observed in rats (27, 28). In agreement with Dicker and co-workers (29), our study demonstrated that this phenomenon could be prevented by yohimbine (an α_2-AR antagonist), suggesting that the reduced lipolytic effect observed at 100 μM adrenaline was probably mediated through the activation of α_2-AR (30). Given the fact that, in *+db/+db* mice, the magnitude of β-ARs–mediated lipolysis was consistently smaller (compared to *+m/+db* mice), our novel findings may provide some clues to the phenomenon of "lipolytic catecholamine resistance," which occurs in human subjects with abdominal obesity (2).

Our results clearly demonstrate that oral folic acid supplementation increased the lipolytic effects induced by β_2- and β_3-ARs activation under the diabetic/obese conditions. The dosages of folic acid used in this study have been shown to be effective in improving endothelial functions in mice-isolated mesenteric artery (14). One of the possible mechanisms responsible for this phenomenon is probably the increase in the expression levels of β_2- and β_3-ARs by folic acid consumption. In fact, alteration of β_3-AR mRNA transcripts in the hyper- and hypo-thyroid rats is responsible for the perturbation of the lipolytic activity (27). Similarly, the abnormally enhanced catecholamine-induced lipolysis in patients with thyrotoxicosis can be restored to normal by anti-thyroid treatment through a down-regulation in the number of β_2-ARs (26). Our study shows that salbutamol (a β_2-AR agonist)–induced lipolysis was enhanced in *+db/+db* mice and was not altered in *+m/+db* mice after the folic acid treatment. Unexpectedly, the protein expressions of β_2-AR in the adipose tissues of both *+m/+db* mice and *+db/+db* mice were decreased, which was inconsistent with the results of the lipolysis response. Reasons for this discrepancy are not known at the moment. It is possible that folic acid (or folate) decreases the abundance of β_2-AR but may increase the activity and/or binding affinity of the β_2-AR. This hypothesis warrants further studies. In contrast to β_2-AR, the protein expression of β_3-AR was consistent with the lipolytic response of the present study. The protein expression of β_3-AR of adipose tissues was increased by folic acid supplementation in *+db/+db* mice but was not altered in *+m/+db* mice. In line with this, folic acid consumption (4 weeks) potentiated the lipolytic effects of β_3-AR agonists (CGP 12177 and BRL 37344) in *+db/+db* mice but not in *+m/+db* mice.

In addition to a change in the expression levels of β-ARs (β_2-AR and β_3-AR), we also considered that an up-regulation or enhanced sensitivity of the downstream cellular signaling pathway of β-ARs may account for the potentiation effects caused by folic acid supplementation on β-ARs–mediated lipolysis in *+db/+db* mice. This notion is supported by our data, which showed that folic acid consumption could enhance the forskolin- (an adenylate cyclase activator) and 8-Br-cAMP (a PKA activator)–induced lipolysis. We did not investigate the mechanism by which folic acid sensitized the cAMP-PKA–dependent pathway of lipolysis in the present study. However, it is tempting to speculate that folic acid supplementation may prevent PKA inactivation (31, 32) and/or enhance the phosphorylation of hormone-sensitive lipase by PKA (3).

It is interesting to point out that the plasma folate levels detected in *+db/+db* mice were significantly lower than those observed in *+m/+db* mice before folic acid consumption. Folic acid supplementation managed to elevate plasma folate levels, as reported (14), in both species. However, the reduced folate levels detected in *+db/+db* mice failed to reach the same/similar levels as observed in *+m/+db* mice after folic acid supplementation. In contrast, folic acid supplementation caused no apparent change in plasma (total) homocysteine levels in both *+m/+db* mice and *+db/+db* mice, suggesting that the alteration of β-ARs–mediated lipolysis and protein expressions of β-ARs in the adipose tissues did not correlate with any change of the plasma homocysteine levels. Although the plasma homocysteine level was not affected by the folic acid treatment, a dose-dependent reduction of plasma resistin level was observed in the *+db/+db* mice after folic acid supplementation (unpublished data), providing direct evidence of the bioavailability of folic acid (or folate) in mice used in this study. In fact, the beneficial effects of folic acid supplementation can be both homocysteine-dependent (11, 33) and homocysteine-independent (14, 15). Our findings demonstrate for the first time that oral folic acid (or folate) supplementation possesses unique modulation effects on the protein expression of β-ARs (namely, β_2- and β_3-ARs in this study) of omental adipose tissues. An altered β-ARs protein expression of the adipose tissues of *+db/+db* mice after folic acid supplementation may be important for, to a certain extent but is not solely responsible for, the change in magnitude of lipolysis *in vitro* of the abdominal (omental) adipose tissues of *+db/+db* mice upon the activation of β-ARs. Thus, our study suggests that folic acid consumption may provide beneficial effects to diabetic patients with obesity.

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1. Frayn KN. Obesity and metabolic disease: is adipose tissue the culprit? Proc Nutr Soc 64:7–13, 2005.
2. Reynisdottir S, Wahrenberg H, Carlstrom K, Rossner S, Arner P. Catecholamine resistance in fat cells of women with upper-body obesity due to decreased expression of β_2-adrenoceptors. Diabetologia 37:428–435, 1994.
3. Carmen GY, Victor SM. Signalling mechanisms regulating lipolysis. Cell Signalling 18:401–408, 2006.
4. de Souza CJ, Burkey BF. β_3-adrenoceptor agonists as anti-diabetic and anti-obesity drugs in humans. Curr Pharm Des 7:1433–1449, 2001.
5. Langin D. Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. Pharmacol Res 53:482–491, 2006.
6. Kim H, Pennisi PA, Gavrilova O, Pack S, Jou W, Setser-Portas J, East-Palmer J, Tang Y, Manganiello VC, Leroith D. Effect of adipocyte β_3-adrenergic receptor activation on the type 2 diabetic MKR mice. Am J Physiol 290:E1227–E1236, 2006.
7. Arch JR, Wilson S. Prospects for β_3-adrenoceptor agonists in the treatment of obesity and diabetes. Int J Obes Relat Metab Disord 20: 191–199, 1996.
8. Hashimoto K, Nagao Y, Ida K, Takeda M, Murakami N, Kato K, Mizota M. Improvement of metabolic disorders and visceral fat obesity by the beta 3-adrenoceptor agonist (R*,R*)-(+/-)-methyl-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl]-phenoxyacetate hydrobromide (BRL35135A) in genetically obese rodents. Biochem Pharmacol 52:1529–1535, 1996.
9. Ntaios GC, Savopoulos CG, Chatzinikolaou AC, Kaiafa GD, Hatzitolios A. Vitamins and stroke: the homocysteine hypothesis still in doubt. Neurologist 14:2–4, 2008.
10. Stanger O, Semmelrock HJ, Wonisch W, Bos U, Pabst E, Wascher TC. Effects of folate treatment and homocysteine lowering on resistance vessel reactivity in atherosclerotic subjects. J Pharmacol Exp Ther 303: 158–162, 2002.
11. Woo KS, Chook P, Lolin YI, Sanderson JE, Metreweli C, Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. J Am Coll Cardiol 34:2002–2006, 1999.
12. Hoogeveen EK, Kostense PJ, Beks PJ, Mackaay AJ, Jakobs C, Bouter LM, Heine RJ, Stehouwer CD. Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease, especially in non–insulin-dependent diabetes mellitus: a population-based study. Arter Thromb Vasc Biol 18:133–138, 1998.
13. Bunout D, Garrido A, Suazo M, Kauffman R, Venegas P, de la Maza P, Petermann M, Hirsch S. Effects of supplementation with folic acid and antioxidant vitamins on homocysteine levels and LDL oxidation in coronary patients. Nutrition 16:107–110, 2000.
14. Clarke ZL, Moat SJ, Miller AL, Randall MD, Lewis MJ, Lang D. Differential effects of low and high dose folic acid on endothelial dysfunction in a murine model of mild hyperhomocysteinaemia. Eur J Pharmacol 551:92–97, 2006.
15. van Etten RW, de Koning EJ, Verhaar MC, Gaillard CA, Rabelink TJ. Impaired NO-dependent vasodilation in patients with type II (non–insulin-dependent) diabetes mellitus is restored by acute administration of folate. Diabetologia 45:1004–1010, 2002.
16. Mahabir S, Ettinger S, Johnson L, Baer DJ, Clevidence BA, Hartman TJ, Taylor PR. Measures of adiposity and body fat distribution in relation to serum folate levels in postmenopausal women in a feeding study. Eur J Clin Nutr 62:644–650, 2008.
17. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444:840–846, 2006.
18. Mural RJ et al. A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. Science 296:1661–1671, 2002.
19. Seto SW, Lam TY, Leung GPH, Au ALS, Ngai SM, Chan SW, Kwan YW. Comparison of vascular relaxation, lipolysis and glucose uptake by peroxisome proliferator–activated receptor-gamma activation in *+db/+m* and *+db/+db* mice. Eur J Pharmacol 572:40–48, 2007.
20. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J Chromatogr 422:43–52, 1987.
21. Morita H, Saito Y, Ohashi N, Yoshikawa M, Katoh M, Ashida T, Kurihara H, Nakamura T, Kurabayashi M, Nagai R. Fluvastatin ameliorates the hyperhomocysteinemia-induced endothelial dysfunction: the antioxidative properties of fluvastatin. Circ J 69:475–480, 2005.
22. Mullick AE, Zaid UB, Athanassious CN, Lentz SR, Rutledge JC, Symons JD. Hyperhomocysteinemia increases arterial permeability and stiffness in mice. Am J Physiol 291:R1349–R1354, 2006.
23. Rodbell M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380, 1964.
24. Harikai N, Hashimoto A, Semma M, Ichikawa A. Characteristics of lipolysis in white adipose tissues of SHR/NDmc-cp rats, a model of metabolic syndrome. Metabolism 56:847–855, 2007.

25. Canova NK, Lincova D, Kmonickova E, Kamenikova L, Farghali H. Nitric oxide production from rat adipocytes is modulated by β_3-adrenergic receptor agonists and is involved in a cyclic AMP-dependent lipolysis in adipocytes. Nitric Oxide 14:200–211, 2006.
26. Lonnqvist F, Wahrenberg H, Hellstrom L, Reynisdottir S, Arner P. Lipolytic catecholamine resistance due to decreased β_2-adrenoceptor expression in fat cells. J Clin Invest 90:2175–2186, 1992.
27. Germack R, Starzec A, Perret GY. Regulation of β_1- and β_3-adrenergic agonist–stimulated lipolytic response in hyperthyroid and hypothyroid rat white adipocytes. Br J Pharmacol 129:448–456, 2000.
28. Germack R, Starzec AB, Vassy R, Perret GY. β-adrenoceptor subtype expression and function in rat white adipocytes. Br J Pharmacol 120: 201–210, 1997.
29. Dicker A, Kaaman M, van Harmelen V, Astrom G, Blanc KL, Ryden M. Differential function of the alpha$_{2A}$-adrenoceptor and phosphodiesterase-3B in human adipocytes of different origin. Int J Obes (Lond) 29: 1413–1421, 2005.
30. Arner P. Human fat cell lipolysis: biochemistry, regulation and clinical role. Best Pract Res Clin Endoc Metab 19:471–482, 2005.
31. Dimon-Gadal S, Gerbaud P, Keryer G, Anderson W, Evain-Brion D, Raynaud F. *In vitro* effects of oxygen-derived free radicals on type I and type II cAMP-dependent protein kinases. J Biol Chem 273:22833–22840, 1998.
32. Raynaud F, Evain-Brion D, Gerbaud P, Marciano D, Gorin I, Liapi C, Anderson WB. Oxidative modulation of cyclic AMP–dependent protein kinase in human fibroblasts: possible role in psoriasis. Free Rad Biol Med 22:623–632, 1997.
33. Racek J, Rusnakova H, Trefil L, Siala KK. The influence of folate and antioxidants on homocysteine levels and oxidative stress in patients with hyperlipidemia and hyperhomocysteinemia. Physiol Res 54:87–95, 2005.