FABP4 is Secreted from Adipocytes by Adenyl Cyclase–PKA– and Guanylyl Cyclase–PKG–Dependent Lipolytic Mechanisms

ARTICLE *in* OBESITY · DECEMBER 2014

Impact Factor: 3.73 · DOI: 10.1002/oby.20954

16 AUTHORS, INCLUDING:

Masato Furuhashi
Sapporo Medical University
51 PUBLICATIONS **3,888** CITATIONS
SEE PROFILE

Hiroshi Akasaka
Sapporo Medical University
52 PUBLICATIONS **417** CITATIONS
SEE PROFILE

Hirofumi Ohnishi
Sapporo Medical University
78 PUBLICATIONS **766** CITATIONS
SEE PROFILE

Hideaki Yoshida
Sapporo Medical University
61 PUBLICATIONS **823** CITATIONS
SEE PROFILE

Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

FABP4 is Secreted from Adipocytes by Adenyl Cyclase-PKA- and Guanylyl Cyclase-PKG-Dependent Lipolytic Mechanisms

Tomohiro Mita[1], Masato Furuhashi[1]*, Shinya Hiramitsu[2], Junnichi Ishii[3], Kyoko Hoshina[1], Shutaro Ishimura[1], Takahiro Fuseya[1], Yuki Watanabe[1], Marenao Tanaka[1], Kohei Ohno[1], Hiroshi Akasaka[1], Hirofumi Ohnishi[1,4], Hideaki Yoshida[1], Shigeyuki Saitoh[1,5], Kazuaki Shimamoto[6], and Tetsuji Miura[1]*

Objective: Fatty acid-binding protein 4 (FABP4) is expressed in adipocytes, and elevated plasma FABP4 levels are associated with obesity-mediated metabolic phenotype. Postprandial regulation and secretory signaling of FABP4 have been investigated.

Methods: Time courses of FABP4 levels were examined during an oral glucose tolerance test (OGTT; $n = 53$) or a high-fat test meal ($n = 35$). Effects of activators and inhibitors of adenyl cyclase (AC)-protein kinase A (PKA) signaling and guanylyl cyclase (GC)-protein kinase G (PKG) signaling on FABP4 secretion from mouse 3T3-L1 adipocytes were investigated.

Results: FABP4 level significantly declined after the OGTT or a high-fat meal, while insulin level was increased. Treatment with low and high glucose concentration or palmitate for 2 h did not affect FABP4 secretion from 3T3-L1 adipocytes. FABP4 secretion was increased by stimulation of lipolysis using isoproterenol, a β_3-adrenoceptor agonist (CL316243), forskolin, dibutyryl-cAMP, and atrial natriuretic peptide, and the induced FABP4 secretion was suppressed by insulin or an inhibitor of PKA (H-89), PKG (KT5823), or hormone sensitive lipase (CAY10499).

Conclusions: FABP4 is secreted from adipocytes in association with lipolysis regulated by AC-PKA- and GC-PKG-mediated signal pathways. Plasma FABP4 level declines postprandially, and suppression of FABP4 secretion by insulin-induced anti-lipolytic signaling may be involved in this decline in FABP4 level.

Obesity (2015) **23**, 359–367. doi:10.1002/oby.20954

Introduction

Fatty acid-binding proteins (FABPs) are approximately 14-15 kDa predominantly cytosolic proteins that can reversibly bind hydrophobic ligands, such as saturated and unsaturated long-chain fatty acids, with high affinity (1,2). FABPs have been proposed to facilitate the transport of lipids to specific compartments in the cell. Among FABPs, fatty acid-binding protein 4 (FABP4), known as adipocyte FABP (A-FABP) or aP2, is mainly expressed in both adipocytes and macrophages and plays an important role in the development of insulin resistance and atherosclerosis (3-6). Furthermore, it has also been demonstrated in experimental models that a small molecule FABP4 inhibitor could be a therapeutic strategy against insulin resistance, diabetes mellitus and atherosclerosis (7).

Recent studies have shown that FABP4 is secreted from adipocytes via a nonclassical secretion pathway (8-11), though there are no typical secretory signal peptides in the sequence of FABP4 (1). It has also been shown that FABP4 acts as an adipokine for the development of hepatic insulin resistance (9) and cardiodepressant effect (12) in experimental models. Furthermore, elevated plasma concentration of FABP4 has been shown to be associated with obesity, insulin resistance, hypertension, cardiac diastolic dysfunction, and atherosclerosis (8,13-19). However, little is known about postprandial regulation of FABP4 level in humans and the signal pathway of FABP4 secretion in the cell. To address these issues, we investigated change in FABP4 levels during an oral glucose tolerance test (OGTT) and a high-fat meal loading test in humans and alterations in FABP4 secretion from 3T3-L1 adipocytes by neurohumoral

[1] Department of Cardiovascular, Renal and Metabolic Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan. Correspondence: Masato Furuhashi (furuhasi@sapmed.ac.jp) [2] Hiramitsu Heart Clinic, Shiroshita-cho 2-35, Nagoya, Aichi, Japan [3] Department of Joint Research Laboratory of Clinical Medicine, Fujita Health University School of Medicine, Toyoake, Aichi, Japan [4] Department of Public Health, Sapporo Medical University School of Medicine, Sapporo, Japan [5] Department of Nursing, Division of Medical and Behavioral Subjects, Sapporo Medical University School of Health Sciences, Sapporo, Japan [6] Sapporo Medical University, Sapporo, Japan.

Funding agencies: M.F. has been supported by grants from JSPS KAKENHI, Uehara Memorial Foundation, SENSHIN Medical Research Foundation, Japan Diabetes Foundation, Takeda Medical Research Foundation, Ono Medical Research Foundation, Takeda Science Foundation, Akiyama Life Science Foundation, and Yamaguchi Endocrine Research Foundation.

Disclosure: The authors declared no conflict of interest.

Author contributions: MF, SH, KS, T.Miura were involved in study design, data analysis, and data interpretation; T.Mita, SH, JI, KH, SI, TF, YW, MT, KO, HA, HO, HY, SS were involved in data collection. All authors were involved in writing the manuscript and approved the final submission.

*These authors equally contributed to this work.

Received: 27 May 2014; **Accepted:** 5 October 2014; **Published online 17 December 2014. doi:10.1002/oby.20954**

factors, which are known to regulate metabolic variables during a postprandial period.

Methods

The present study consisted of two human studies (Study 1 and Study 2) and one *in vitro* study (Study 3). Both human studies strictly conformed to the principles in the Declaration of Helsinki, and Study 1 and Study 2 were approved by the Ethical Committee of Sapporo Medical University and the Ethical Committee of Fujita Health University, respectively. Written informed consent was received from all of the study subjects. Study 3 was approved by the Animal Care and Experiment Committee of Sapporo Medical University.

Study 1: FABP4 concentration during OGTT

In Study 1, we recruited 615 Japanese subjects (males/females: 264/351, mean age: 64.9 years) from residents of Sobetsu Town in Hokkaido, the northernmost island of Japan, who participated in the Tanno-Sobetsu Study, a study with a population-based cohort design, in 2010. Medical examinations were performed between 06:00 h and 09:00 h after an overnight fast. After measuring anthropometric parameters, blood pressure was measured twice consecutively on the upper arm using an automated sphygmomanometer (HEM-907, Omron, Kyoto, Japan) in a seated resting position, and average blood pressure was used for analysis. Body mass index (BMI) was calculated as body weight (in kilograms) divided by the square of body height (in meters). Peripheral venous blood samples were obtained after physical examination for biochemical analyses, and plasma samples were analyzed immediately or stored at −80°C until biochemical analyses.

If the examination showed that fasting glucose was 100–125 (mg/dl) and/or hemoglobin A1c (HbA1c) expressed in national glycohemoglobin standardization program (NGSP) scale was 5.6-6.4%, the subject was invited to undergo a 75 g OGTT scheduled one month after the annual examination. A total of 53 subjects (males/females: 25/28, mean age: 66.0 years) accepted the invitation and underwent 75 g OGTTs. In the OGTT, blood samples were collected before and at 1 and 2 h after ingestion of Trelan-G^{TM} (75 g glucose in 225 ml water) for determination of plasma levels of glucose, insulin and FABP4.

Study 2: FABP4 concentration during a high-fat meal loading test

Of patients who visited outpatient clinics affiliated with Fujita Health University, 35 study subjects were selected on the basis of entry criteria (age of 33–53 years, no treatment with lipid-lowering agents or oral anti-diabetic agents for at least 12 weeks) and exclusion criteria (serious hepatic disease, renal disease, or atherosclerosis). After overnight fasting for at least 12 h, subjects were given a high-fat meal for breakfast. A fast-food meal that included a hamburger with sausage and egg, an apple pie and Coca-Cola® was chosen as the high-fat test meal (1001 kcal; protein, 31.6 g; lipids, 61.4 g; carbohydrate, 79.8 g; cholesterol, 299 mg) as previously reported (20). The subjects were requested to eat the test meal in 20–30 min. At 0, 2, 4, and 6 h after finishing the test meal, blood samples were collected for determination of plasma levels of glucose, insulin, triglycerides, and FABP4.

Measurements

Plasma concentration of FABP4 was measured using a commercially available enzyme-linked immunosorbent assay kit for FABP4 (Biovendor R&D, Modrice, Czech Republic). The accuracy, precision, and reproducibility of the kit have been described previously (8). The intra- and interassay coefficient variances in the kits were $< 5\%$.

Plasma glucose was determined by the glucose oxidase method. Fasting plasma insulin was measured by a radioimmunoassay method (Insulin RIA bead, Dianabot, Tokyo, Japan). Creatinine (Cr), aspartate transaminase (AST), alanine aminotransferase (ALT), and lipid profiles, including total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides, were determined by enzymatic methods. Low-density lipoprotein (LDL) cholesterol level was calculated by the Friedewald equation. Hemoglobin A1c (HbA1c) was determined by a latex coagulation method and was expressed in NGSP scale. As an index of renal function, estimated glomerular filtration rate (eGFR) was calculated by an equation for Japanese (21): eGFR (ml/min/1.73 m^2) = $194 \times Cr^{(-1.094)} \times age^{(-0.287)} \times 0.739$ (if female). HOMA-R, an index of insulin resistance, was calculated by the previously reported formula: insulin (μU/ml) × glucose (mg/dl)/405.

Study 3: Secretion of FABP4 from 3T3-L1 adipocytes

All biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Pre-adipocyte 3T3-L1 cells were obtained from Health Science Research Resources Bank (Osaka, Japan) and were maintained and propagated in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% cosmic calf serum (CCS) (Hyclone, Logan, UT), 50 U/ml penicillin and 50 mg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. Differentiation of 3T3-L1 cells was initiated by incubation in an induction medium (1 μM dexamethasone, 0.5 mM isobutylmethyl xanthine, 1 μM troglitazone, and 5 μg/ml insulin). Following a 4-day induction period (two 48-h incubations), the medium was changed to a post-induction medium (1 μM troglitazone and 5 μg/ml insulin) for an additional 2 days, followed by a medium supplemented with 0.5 μg/ml of insulin for 2 days. Thereafter, the medium was replaced with the maintaining medium.

After overnight serum depletion by 0.5% bovine serum albumin (BSA) in DMEM, the differentiated 3T3-L1 adipocytes were treated with a low (1 mg/ml) or high (4.5 mg/ml) concentration of glucose, insulin, palmitate, isoproterenol, dibutyryl-cAMP (db-cAMP), H-89, CL316243, forskolin, atrial natriuretic peptide (ANP) (Peptide Institute, Osaka, Japan), KT5823 (Millipore, Billerica, MA), and CAY10499 (Cayman, Ann Arbor, MI) in DMEM supplemented with 0.5% BSA for 2 h. The doses of reagents and incubation periods varied according to the experimental protocol. Each experiment was done in at least triplicate.

The conditioned medium (CM) from adipocytes was filtered to obtain a 10–50 kDa fraction of proteins using Amicon Ultra 10K and 50K devices (Millipore).

Total protein content of the cell lysate (CL) in a cell lysis buffer, containing 50 mM Tris-HCl (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 10 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 40 mM β-glycerophosphate, 0.5% NP-40, and 1% protease inhibitor cocktail,

TABLE 1 Characteristics of the studied subjects (Study 1)

	Total	Male	Female
n	53	25	28
Age (years)	66.0 ± 1.3	67.3 ± 1.6	64.9 ± 2.1
Body mass index (kg/m^2)	23.4 ± 0.5	24.3 ± 0.5	22.5 ± 0.8
Waist circumference (cm)	85.7 ± 1.5	89.0 ± 1.5	82.7 ± 2.4*
Systolic blood pressure (mmHg)	140.2 ± 3.1	146.2 ± 4.1	134.7 ± 4.3
Diastolic blood pressure (mmHg)	77.9 ± 1.6	81.8 ± 2.1	74.4 ± 2.1*
Medication			
ACE inhibitor	1 (1.9)	0 (0)	1 (3.6)
Angiotensin II receptor blocker	6 (11.3)	2 (8)	4 (14.3)
Calcium channel blocker	12 (22.6)	2 (8)	10 (35.7)
β-Blocker	4 (7.5)	2 (8)	2 (7.1)
Antiplatelet drug	2 (3.8)	2 (8)	0 (0)
Statin	11 (20.8)	3 (12)	8 (28.6)
Biochemical data			
Total cholesterol (mg/dl)	215.1 ± 4.0	207.3 ± 5.6	222.1 ± 5.3
HDL cholesterol (mg/dl)	67.9 ± 2.5	57.9 ± 2.9	76.8 ± 3.2*
LDL cholesterol (mg/dl)	124.2 ± 3.7	123.4 ± 5.2	125.0 ± 5.2
Triglycerides (mg/dl)	115.1 ± 9.1	130.1 ± 15.8	101.6 ± 9.5
Glucose (mg/dl)	96.4 ± 1.2	98.1 ± 1.8	95.0 ± 1.6
HbA1c (%)	5.67 ± 0.03	5.67 ± 0.05	5.68 ± 0.03
Insulin (μU/ml)	6.3 ± 0.8	6.8 ± 1.4	6.0 ± 0.9
HOMA-R	1.57 ± 0.21	1.70 ± 0.37	1.46 ± 0.23
Cr (mg/dl)	0.74 ± 0.02	0.83 ± 0.02	0.67 ± 0.01*
eGFR (ml/min/1.73 m^2)	70.8 ± 1.5	72.8 ± 2.3	68.9 ± 1.8
AST (IU/l)	24.8 ± 0.8	25.4 ± 1.4	24.1 ± 0.9
ALT (IU/l)	23.3 ± 1.5	25.9 ± 2.7	21.0 ± 1.3

Variables are expressed as *n* (%) or means ± SEM. *$P < 0.05$ vs. male.

was assessed by a microplate protein assay based on Lowry method (Bio-Rad, Hercules, CA). For extraction of exosome and microvesicle (EM) fraction, the CM was first centrifuged at 300*g* for 10 min and then at 16,500*g* for 20 min to remove cell debris and aggregates. The supernatant was ultracentrifuged at 120,000*g* for 70 min. Pelleted vesicles were resuspended in phosphate-buffered saline and ultracentrifuged again for washing at 120,000*g* for 15 min. The pelleted vesicles were resuspended in the cold lysis buffer.

For Western blot analysis, equal amounts of protein per CL sample, the filtered CM, the EM and non-EM fraction of CM and known molecular weight markers were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred onto PVDF membranes (Whatman, Florham Park, NJ) and incubated for 1 h at room temperature with a blocking solution of 3% BSA in Tris-buffered saline buffer containing 0.1% Tween 20 (TBST). The blocked membranes were incubated with primary antibodies for FABP4 (Abcam, Tokyo, Japan), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) and caveolin-1 (Cell Signaling, Danvers, MA) overnight at 4°C and washed three times with TBST. The membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at room temperature and washed. Immunodetection analyses were performed using an enhanced chemiluminescence kit (Roche Diagnostics, Tokyo, Japan).

For lipolysis analysis, release of free fatty acids into the CM was quantitated by a colorimetric assay (Wako Pure Chemical Industries, Osaka, Japan), and data were normalized to total cellular protein content and expressed as the fold changes from basal levels.

Statistical analysis

Numeric variables are expressed as means ± SEM. Comparison between two groups was performed with an unpaired Student's *t*-test for normally distributed data or Mann-Whitney's *U*-test for skewed variables. One-way repeated measures ANOVA was used for testing differences in time courses of parameters. One-way analysis of variance and Tukey-Kramer *post hoc* test were used for detecting significant differences in data between 3 groups. A *P* value of less than 0.05 was considered statistically significant. All data were analyzed by using JMP 9 for Macintosh (SAS Institute, Cary, NC).

Results

Study 1

Basal characteristics of the subjects are shown in Table 1. The proportion of subjects on medication including anti-hypertensive and/or anti-dyslipidemic drugs was 39.6%. Male subjects had significantly larger waist circumference and higher levels of diastolic blood pressure and Cr and lower level of HDL cholesterol than did female

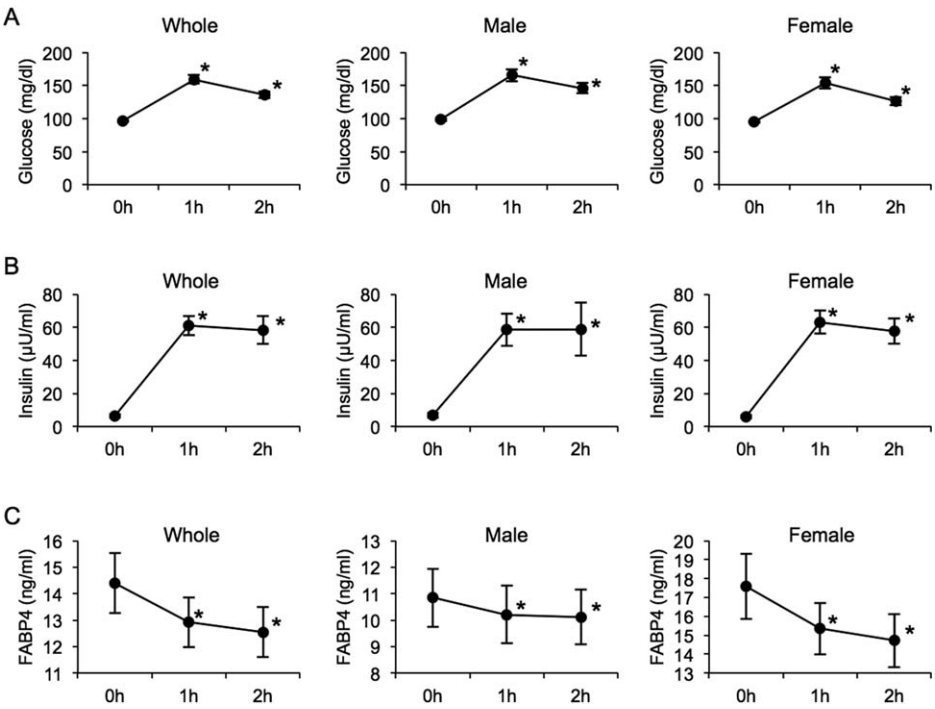

Figure 1 Time courses of plasma levels of (**A**) glucose, (**B**) insulin, and (**C**) FABP4 during the 75 g oral glucose tolerance test (OGTT) in 53 subjects (males/females: 25/28) recruited from the Tanno-Sobetsu cohort study. *$P < 0.01$ vs. at 0 h time point.

subjects. During the OGTT, glucose and insulin levels were significantly increased to 159.2 ± 6.1 mg/dl and 60.9 ± 5.8 μU/ml, respectively, and remained higher at 2 h after glucose loading than baseline values (Figure 1A, B), whereas FABP4 level was consistently decreased to 12.9% of the baseline value (Figure 1C).

Study 2

Basal characteristics of the subjects are shown in Table 2. Mean age, BMI, and waist circumference of the 35 recruited male subjects were 40.2 ± 0.9 years, 25.8 ± 0.5 kg/m^2, and 88.5 ± 1.1 cm, respectively. After a high-fat meal, glucose level gradually decreased until 4 h after the meal and then slightly recovered (Figure 2A). Insulin level was increased to 28.9 ± 4.3 μU/ml at 2 h after the meal and then gradually decreased (Figure 2B). Triglycerides level increased and plateaued at 4 h after the meal (Figure 2C). FABP4 level was consistently decreased to 20.7% of the baseline value during a 4-h period after the meal and then showed a slight trend for recovery (Figure 2D).

Study 3

Based on data in Study 1 and Study 2, we hypothesized that changes in plasma glucose, insulin, and/or lipids during glucose and high-fat meal loading tests are associated with down-regulation of FABP4 secretion, leading to reduction in FABP4 level. Hence, differentiated 3T3-L1 adipocytes were treated with several stimuli for 2 h, including low (1 mg/ml) and high (4.5 mg/ml) concentration of glucose, 0.5 μg/ml insulin, 1 mM palmitate, 10 μM isoproterenol, a pan-β-adrenergic agonist, as an agent for counteracting insulin-mediated anti-lipolytic action, and 0.5 mM db-cAMP, a cAMP analogue as a protein kinase A (PKA) activator of downstream signaling of the β-adrenergic receptor. Western blot analysis showed that FABP4 was present in both the CL and CM of 3T3-L1 adipocytes and that GAPDH, a non-secretory protein, was not present in the CM (Figure 3A). The results indicate that FABP4 in the CM is a result of its secretion from 3T3-L1 adipocytes, not a result of its leakage via injured cell membranes.

Treatment with a low or high concentration of glucose, insulin, or palmitate for 2 h did not affect FABP4 secretion from 3T3-L1 adipocytes (Figure 3A). However, secretion of FABP4 was increased by treatment with isoproterenol or db-cAMP (Figure 3A). The effects of isoproterenol and db-cAMP on FABP4 secretion from adipocytes were dose-dependent (Figure 3B, C). Isoproterenol- and db-cAMP-induced FABP4 secretion was inhibited by co-treatment with insulin, which converts cAMP to 5'-AMP (Figure 3D, E). Isoproterenol-induced FABP4 secretion was also suppressed by co-treatment with H-89, a PKA inhibitor (Figure 3F).

A selective β_3-adrenergic agonist, CL316243, increased FABP4 secretion in a dose-dependent manner (Figure 3G), and the effect of CL316243 was attenuated by insulin or H-89 (Figure 3H). Forskolin, an activator of adenyl cyclase (AC), increased FABP4 secretion, and the increased secretion was inhibited by insulin or H-89 (Figure 3I, J).

Other than β-adrenergic receptor-mediated AC-PKA signaling, ANP, an activator of natriuretic peptide receptor-A (NPR-A)-mediated guanylyl cyclase (GC)-protein kinase G (PKG) signaling, also

TABLE 2 Characteristics of the studied male subjects (Study 2)

n	35
Age (years)	40.2 ± 0.9
Body mass index (kg/m^2)	25.8 ± 0.5
Waist circumference (cm)	88.5 ± 1.1
Systolic blood pressure (mmHg)	129.5 ± 2.9
Diastolic blood pressure (mmHg)	79.1 ± 2.8
Medication	
ACE inhibitor	0
Angiotensin II receptor blocker	3 (8.6)
Calcium channel blocker	2 (5.7)
β-Blocker	0
Antiplatelet drug	0
Statin	0
Biochemical data	
Total cholesterol (mg/dl)	226.2 ± 5.5
HDL cholesterol (mg/dl)	55.1 ± 2.0
LDL cholesterol (mg/dl)	137.5 ± 4.5
Triglycerides (mg/dl)	181.1 ± 14.4
Glucose (mg/dl)	101.9 ± 3.0
HbA1c (%)	5.8 ± 0.2
Insulin (μU/ml)	9.2 ± 1.0
HOMA-R	2.33 ± 0.27
Cr (mg/dl)	0.80 ± 0.02
eGFR (ml/min/1.73 m^2)	87.4 ± 2.4
AST (IU/l)	23.6 ± 1.2
ALT (IU/l)	30.5 ± 4.1

Variables are expressed as *n* (%) or means ± SEM.

increased FABP4 secretion in a dose-dependent manner (Figure 3K). KT5823, a PKG inhibitor, suppressed ANP-mediated FABP4 secretion from 3T3-L1 adipocytes (Figure 3L).

At the last step of lipolysis, FABP4 secretion in response to both PKA and PKG signals by isoproterenol and ANP, respectively, was inhibited by CAY10499, an inhibitor of hormone-sensitive lipase (HSL) (Figure 3M, N). Furthermore, lipolysis from adipocytes shown by release of fatty acids into the CM was augmented by activators of AC-PKA or GC-PKG signal pathways, and the induction of lipolysis was inhibited by inhibitors of AC-PKA, GC-PKG or HSL (Figure 4A-F).

FABP4 was released via both the EM and non-EM fractions of CM by treatment with isoproterenol, but the amount of FABP4 secretion in the EM fraction was smaller than that in the non-EM fraction (Figure 5A). On the other hand, the EM-mediated FABP4 secretion was scarcely detected by treatment with ANP (Figure 5B).

Discussion

The main finding of the present study was that FABP4 was secreted from adipocytes under regulation by lipolytic signal pathways, and our current hypothesis regarding regulatory mechanisms of FABP4 secretion from adipocytes is shown in Figure 6. There are two distinct signal pathways of activation of lipolysis by phosphorylation of HSL: β-adrenergic receptor-mediated AC-PKA and NPR-A-mediated GC-PKG pathways (22,23). Activation of AC-PKA signaling by isoproterenol, CL316243, forskolin, or db-cAMP and activation of GC-PKG signaling by ANP increased secretion of FABP4 from adipocytes. Conversely, induced FABP4 secretion was decreased by inhibition of AC-PKA signaling by insulin or H-89, inhibition of GC-PKG signaling by KT5823 and inhibition of HSL

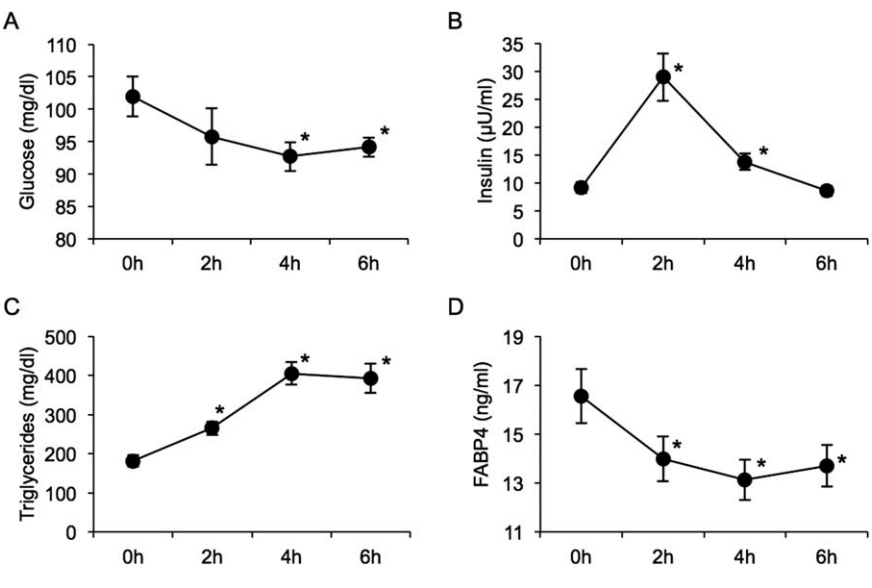

Figure 2 Time courses of plasma levels of (**A**) glucose, (**B**) insulin, (**C**) triglycerides, and (**D**) FABP4 after eating a high-fat test meal in 35 male subjects recruited from clinics. *$P < 0.01$ vs. at 0 h time point.

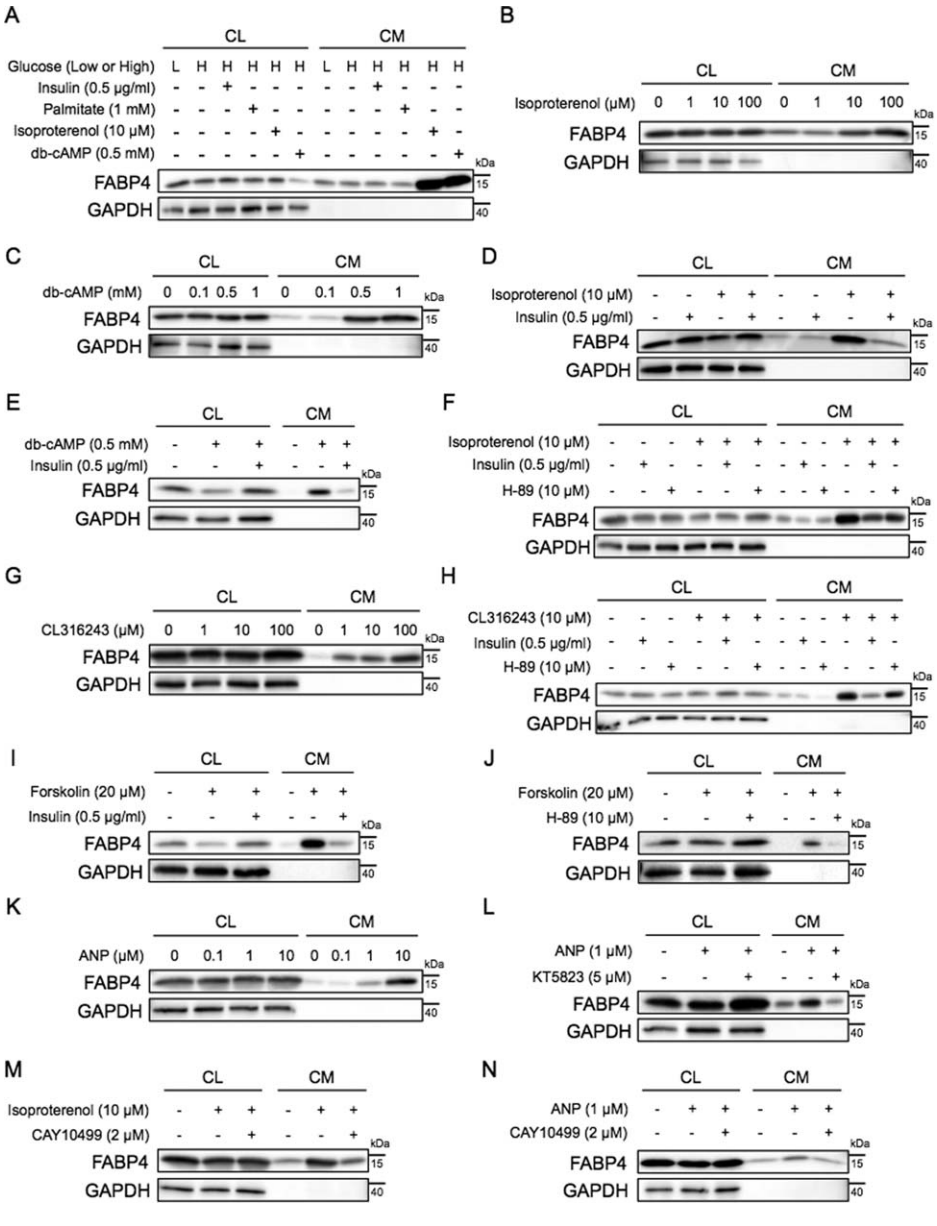

Figure 3 FABP4 secretion from 3T3-L1 adipocytes. Western blot analysis using the cell lysate (CL) and conditioned medium (CM) of 3T3-L1 adipocytes treated with several agents for 2 h: (**A**) low (L; 1 mg/ml) and high (H; 4.5 mg/ml) concentration of glucose, 0.5 μg/ml insulin, 1 mM palmitate, 10 μM isoproterenol, and 0.5 mM db-cAMP; (**B**) 0–100 μM isoproterenol; (**C**) 0–1 mM db-cAMP; (**D**) 10 μM isoproterenol and 0.5 μg/ml insulin; (**E**) 0.5 mM db-cAMP and 0.5 μg/ml insulin; (**F**) 10 μM isoproterenol, 0.5 μg/ml insulin, and 10 μM H-89; (**G**) 0–100 μM CL316243; (**H**) 10 μM CL316243, 0.5 μg/ml insulin, and 10 μM H-89; (**I**) 20 μM forskolin and 0.5 μg/ml insulin; (**J**) 20 μM forskolin and 10 μM H-89; (**K**) 0–10 ANP (μM); (**L**) 1 μM ANP and 5 μM KT5823; (**M**) 10 μM isoproterenol and 2 μM CAY10499; and (**N**) 1 μM ANP and 2 μM CAY10499.

by CAY10499. Of note, it was previously reported that lipolysis is mediated in part through the interaction of FABP4 with HSL in adipocytes (24-26). Collectively, the present results indicate the presence of a dual regulatory mechanism of FABP4 secretion in adipocytes. FABP4 may be a carrier protein for transport of fatty acids generated by lipolysis from lipid droplets to extracellular and/or intracellular utilization (Figure 6).

The present study showed for the first time that FABP4 level declines postprandially in humans. This finding is consistent with the results of a previous study in mice showing that refeeding after a 24-h fast decreased plasma FABP4 concentration without change in FABP4 protein expression in adipose tissue (9). To identify the metabolic parameters involved in the regulation of plasma FABP4, we employed two different types of calorie

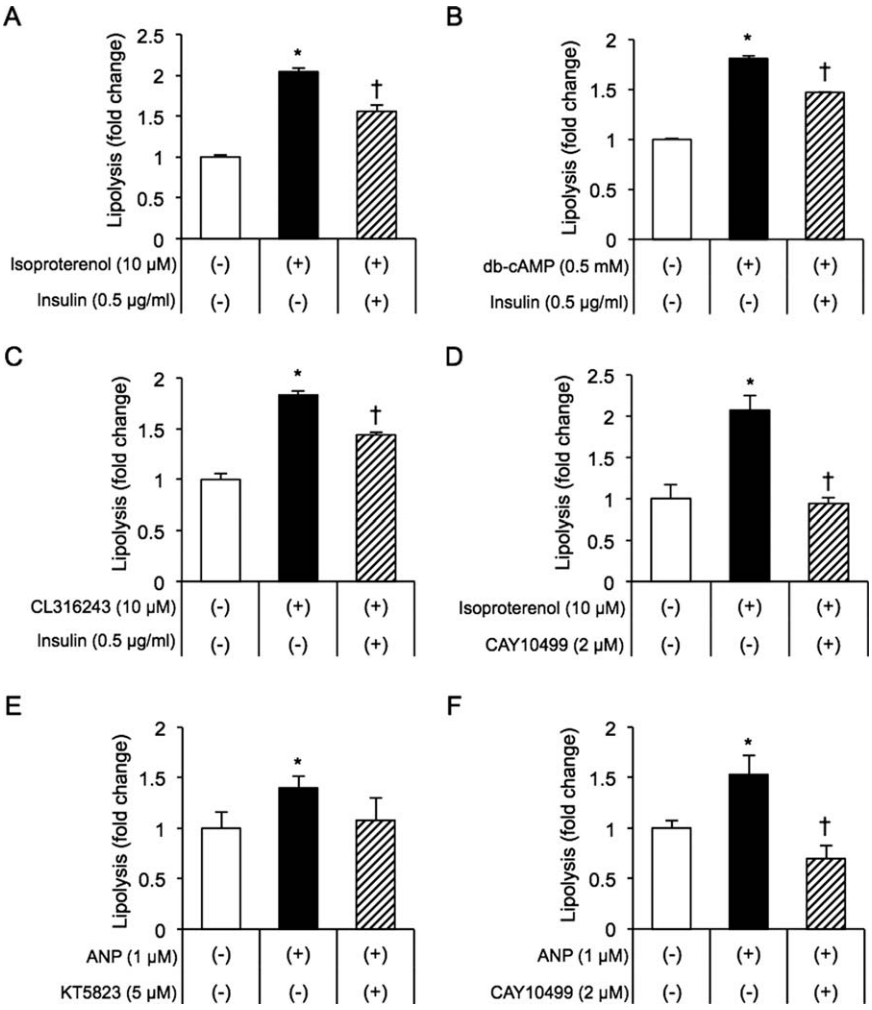

Figure 4 Lipolysis analysis assessed by release of free fatty acids into the medium of 3T3-L1 adipocytes treated with several agents for 2 h: (**A**) 10 μM isoproterenol and 0.5 μg/ml insulin; (**B**) 0.5 mM db-cAMP and 0.5 μg/ml insulin; (**C**) 10 μM CL316243 and 0.5 μg/ml insulin; (**D**) 10 μM isoproterenol and 2 μM CAY10499; (**E**) 1 μM ANP and 5 μM KT5823; and (**F**) 1 μM ANP and 2 μM CAY10499. Each treatment group ($n = 3$∼5). Data were normalized to total cellular protein content and expressed as the fold changes from basal levels. *$P < 0.05$ vs. nonstimulation, †$P < 0.05$ vs. single stimulation.

loading tests: OGTT and high-fat diet. During the OGTT and high-fat meal loading test, directions of change in glucose levels were opposite. However, insulin level was similarly elevated during both the OGTT and meal loading test, and triglycerides were also elevated after high-fat meal loading. The results suggest that insulin and/or lipids, but not plasma glucose, modulate plasma FABP4 level. In the present *in vitro* experiments, treatment with palmitate, a fatty acid, for 2 h did not change secretion of FABP4 from 3T3-L1 adipocytes, though it has been reported that long-term stimulation of adipocytes with fatty acids increases expression of FABP4 (27). In contrast, treatment with insulin markedly suppressed induction of FABP4 secretion from 3T3-L1 adipocytes. These findings support the notion that reduction in FABP4 levels during the OGTT and high-fat meal loading test is because of decreased FABP4 secretion via augmented anti-lipolytic signaling activated by increased insulin.

FABP4 lacks an N-terminal secretory signal sequence (1), which is necessary for the classical secretory pathway, i.e., endoplasmic reticulum (ER)-Golgi-dependent pathway. However, proteins can also be actively secreted from eukaryotic cells via the ER–Golgi-independent pathways (28). Previous studies demonstrated that FABP4 secretion was not influenced by treatment with inhibitors of protein secretion, brefeldin and monensin that block vesicular traffic at the ER and the Golgi apparatus, indicating a nonclassical secretion mechanism for FABP4 (9,10). On the other hand, FABP4 secretion from human adipocytes was shown to be increased by ionomycin, an ionophore that raises intracellular Ca^{2+} levels, indicating contribution of a calcium-dependent and nonclassical secretory mechanism (11). It has also been shown that FABP4 is secreted partially by adipocyte-derived microvesicles (10), an established mechanism for unconventional secretion from adipocytes (28). Furthermore, release of a small fraction of FABP4 via microvesicles by isolated mature human

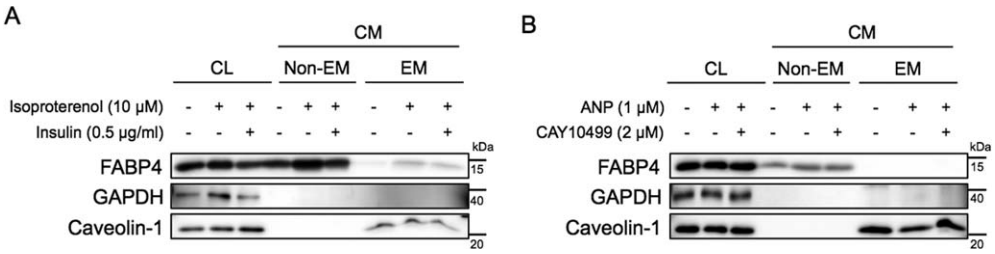

Figure 5 Exosome and microvesicle-mediated FABP4 secretion from 3T3-L1 adipocytes. Western blot analysis using the cell lysate (CL) and conditioned medium (CM), including the exosome and microvesicle (EM) and non-EM fractions, of 3T3-L1 adipocytes treated with several agents for 2 h: (**A**) 10 μM isoproterenol and 0.5 μg/ml insulin and (**B**) 1 μM ANP and 2 μM CAY10499.

adipocytes has been demonstrated (12). We confirmed that FABP4 was mainly secreted via the non-EM fraction rather than the EM fraction (Figure 5). It has previously been reported that the microvesicles isolated from adipocytes only had a minor cardiodepressant activity in accordance with low FABP4 content, whereas microvesicle-free supernatant showed a significant cardiodepressant effect along with large amounts of FABP4 (12). Both microvesicle-free-mediated and microvesicle-secreted FABP4 were also down-regulated by insulin as similarly demonstrated in the present study and up-regulated by ionomycin (10). In the present study, EM-mediated FABP4 secretion by ANP was scarcely detected. It may be due to less secretion of FABP4 compared with isoproterenol stimulation. Taken together, these results suggest that FABP4 is actively released by unconventional mechanisms and by adipocyte-derived exosomes and microvesicles from

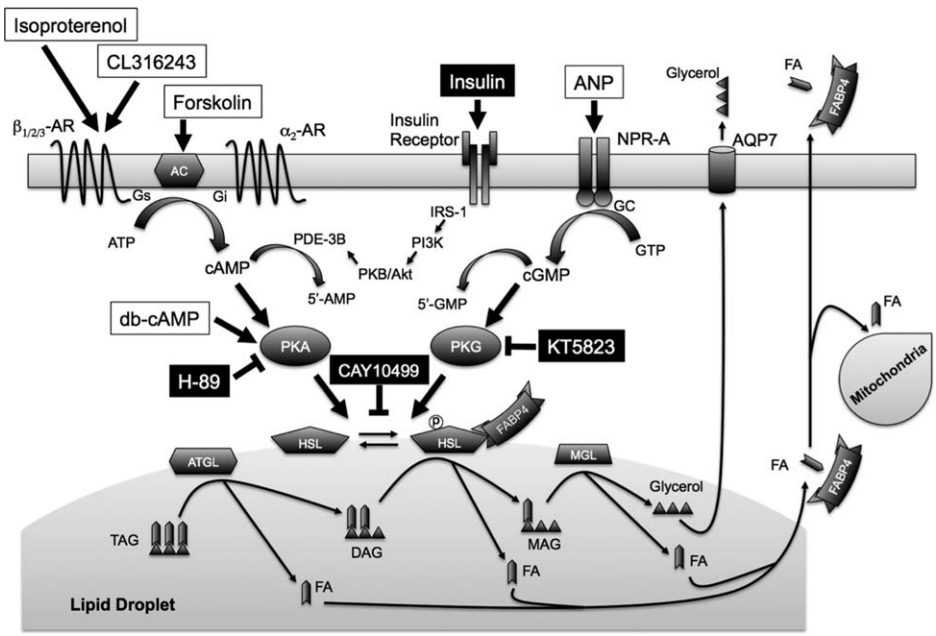

Figure 6 Possible mechanism of FABP4 secretion from adipocytes in association with lipolysis. Two distinct signal pathways of activation of lipolysis by phosphorylation of hormone-sensitive lipase (HSL), including β-adrenergic receptor-mediated adenyl cyclase (AC)-protein kinase A (PKA) and natriuretic peptide receptor-A (NPR-A)-mediated guanylyl cyclase (GC)-protein kinase G (PKG) pathways, are shown. FABP4 has been reported to directly interact with HSL for regulation of its activity. Activation of lipolysis signaling in both AC-PKA and GC-PKG pathways increased FABP4 secretion in the present study. FABP4 may be a carrier protein for transport of fatty acids (FA) generated by lipolysis from lipid droplets to extracellular and/or intracellular utilization. The following agents were used in Study 3: isoproterenol, a pan-β-adrenergic agonist; CL316243, a selective β_3-adrenergic agonist; forskolin, an AC activator; db-cAMP, an analog of cAMP; H-89, a PKA inhibitor; atrial natriuretic peptide (ANP), a NPR-A agonist; KT5823, a PKG inhibitor; and CAY10499, a HSL inhibitor. AR, adrenergic receptor; ATGL, adipose triglyceride lipase; AQP7, aquaporin 7; DAG, diacylglyceride; MAG, monoacylgyceride; MGL, monoacylglycerol lipase; TAG, triacylglyceride; IRS-1, insulin receptor substrate 1; PDE-3B, phosphodiesterase 3B; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B.

adipocytes by AC-PKA- and GC-PKG-dependent signal pathways and/or an intracellular calcium-dependent mechanism. Relationships between PKA, PKG, and other calcium regulatory mechanisms in regulation of extracellular release of FABP4 from adipocytes may warrant further investigations.

Previous studies using animal models indicated that FABP4 plays a significant role in several aspects of metabolic syndrome, including insulin resistance, type 2 diabetes, and atherosclerosis, through its action at the interface of metabolic and inflammatory pathways in adipocytes and macrophages (1-6). In humans, increased plasma FABP4 levels were associated with obesity, insulin resistance, hypertension, cardiac dysfunction, and atherosclerosis (8,13-19). As evidence supporting extracellular roles of FABP4 as an adipokine, direct effects of FABP4 have been demonstrated on hepatic glucose production *in vivo* and *in vitro* (9) and on cardiomyocyte contraction *in vitro* (12). The present results showing an active and regulated secretory process of FABP4 add supportive information to accumulating evidence of an important role of extracellular FABP4 in the pathogenesis of metabolic and cardiovascular complications of obesity.

The present study has limitations. First, 39.6% and 14.2% of the subjects in Study 1 and Study 2, respectively, had been treated with drugs, including stains and angiotensin II receptor blockers, which were reported to modulate FABP4 concentrations (29,30). Therefore, FABP4 data might be modulated by such drugs. Second, the subjects were not randomly selected from a population but were enrolled on voluntary basis. Thus, the possibility of selection bias in data in Study 1 and Study 2, including FABP4 data, cannot be excluded. Third, the number of subjects in the present study was relatively small, leaving a possibility of type II errors in statistical analyses. Interventional studies using larger numbers of subjects not treated with any medication are necessary for critically determining whether regulation of FABP4 level is associated with lipolytic pathways in humans. Fourth, the recruited subjects were younger in Study 2 than in Study 1. A direct relationship between FABP4 secretion and aging remains unclear. This issue warrants further investigation using a longitudinal approach.

In conclusion, FABP4 is secreted from adipocytes in association with lipolysis regulated by AC-PKA and GC-PKG signal pathways. Plasma FABP4 level declines postprandially in humans presumably because of reduced FABP4 secretion via anti-lipolytic signaling of increased insulin. An understanding of the secretory mechanism of FABP4 from adipocytes may enable the development of new therapeutic strategies for cardiovascular and metabolic diseases. O

Acknowledgments

We thank Ms. Shiho Ishikawa for technical help and members of Fujita Ezetimibe Study Assembly (FESTA) investigators group for their cooperation in collection of data.

References

1. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov* 2008;7:489-503.
2. Furuhashi M, Ishimura S, Ota H, Miura T. Lipid chaperones and metabolic inflammation. *Int J Inflam* 2011;2011:642612.
3. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996;274:1377-1379.
4. Makowski L, Boord JB, Maeda K, et al. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med* 2001;7:699-705.
5. Furuhashi M, Fucho R, Gorgun CZ, Tuncman G, Cao H, Hotamisligil GS. Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest* 2008;118:2640-2650.
6. Boord JB, Maeda K, Makowski L, et al. Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice. *Circulation* 2004;110:1492-1498.
7. Furuhashi M, Tuncman G, Gorgun CZ, et al. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature* 2007;447:959-965.
8. Xu A, Wang Y, Xu JY, et al. Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. *Clin Chem* 2006;52:405-413.
9. Cao H, Sekiya M, Ertunc ME, et al. Adipocyte lipid chaperone AP2 is a secreted adipokine regulating hepatic glucose production. *Cell Metab* 2013;17:768-778.
10. Kralisch S, Ebert T, Lossner U, Jessnitzer B, Stumvoll M, Fasshauer M. Adipocyte fatty acid-binding protein is released from adipocytes by a non-conventional mechanism. *Int J Obes* 2014;38:1251-1254.
11. Schlottmann I, Ehrhart-Bornstein M, Wabitsch M, Bornstein SR, Lamounier-Zepter V. Calcium-dependent release of adipocyte fatty acid binding protein from human adipocytes. *Int J Obes* 2014;38:1221-1227.
12. Lamounier-Zepter V, Look C, Alvarez J, et al. Adipocyte fatty acid-binding protein suppresses cardiomyocyte contraction: A new link between obesity and heart disease. *Circ Res* 2009;105:326-334.
13. Xu A, Tso AW, Cheung BM, et al. Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: A 5-year prospective study. *Circulation* 2007;115:1537-1543.
14. Tso AW, Xu A, Sham PC, et al. Serum adipocyte fatty acid binding protein as a new biomarker predicting the development of type 2 diabetes: A 10-year prospective study in a Chinese cohort. *Diabetes Care* 2007;30:2667-2672.
15. von Eynatten M, Breitling LP, Roos M, Baumann M, Rothenbacher D, Brenner H. Circulating adipocyte fatty acid-binding protein levels and cardiovascular morbidity and mortality in patients with coronary heart disease: A 10-year prospective study. *Arterioscler Thromb Vasc Biol* 2012;32:2327-2335.
16. Ota H, Furuhashi M, Ishimura S, et al. Elevation of fatty acid-binding protein 4 is predisposed by family history of hypertension and contributes to blood pressure elevation. *Am J Hypertens* 2012;25:1124-1130.
17. Ishimura S, Furuhashi M, Watanabe Y, et al. Circulating levels of fatty acid-binding protein family and metabolic phenotype in the general population. *PLoS ONE* 2013;8:e81318.
18. Furuhashi M, Ishimura S, Ota H, et al. Serum fatty acid-binding protein 4 is a predictor of cardiovascular events in end-stage renal disease. *PLoS ONE* 2011;6:e27356.
19. Fuseya T, Furuhashi M, Yuda S, et al. Elevation of circulating fatty acid-binding protein 4 is independently associated with left ventricular diastolic dysfunction in a general population. *Cardiovasc Diabetol* 2014;13:126.
20. Hiramitsu S, Miyagishima K, Ishii J, et al. Effect of ezetimibe on lipid and glucose metabolism after a fat and glucose load. *J Cardiol* 2012;60:395-400.
21. Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 2009;53:982-992.
22. Stich V, Berlan M. Physiological regulation of NEFA availability: Lipolysis pathway. *Proc Nutr Soc* 2004;63:369-374.
23. Moro C, Lafontan M. Natriuretic peptides and cGMP signaling control of energy homeostasis. *Am J Physiol Heart Circ Physiol* 2013;304:H358-368.
24. Shen WJ, Sridhar K, Bernlohr DA, Kraemer FB. Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. *Proc Natl Acad Sci U S A* 1999;96:5528-5532.
25. Scheja L, Makowski L, Uysal KT, et al. Altered insulin secretion associated with reduced lipolytic efficiency in aP2-/- mice. *Diabetes* 1999;48:1987-1994.
26. Coe NR, Simpson MA, Bernlohr DA. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J Lipid Res* 1999;40:967-972.
27. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: Tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 1994;8:1224-1234.
28. Nickel W, Rabouille C. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 2009;10:148-155.
29. Karpisek M, Stejskal D, Kotolova H, et al. Treatment with atorvastatin reduces serum adipocyte-fatty acid binding protein value in patients with hyperlipidaemia. *Eur J Clin Invest* 2007;37:637-642.
30. Miyoshi T, Doi M, Hirohata S, et al. Olmesartan reduces arterial stiffness and serum adipocyte fatty acid-binding protein in hypertensive patients. *Heart Vessels* 2011;26:408-413.