

Adipocyte Fatty Acid–Binding Protein Suppresses Cardiomyocyte Contraction

A New Link Between Obesity and Heart Disease

Valéria Lamounier-Zepter, Christiane Look, Julio Alvarez, Torsten Christ, Ursula Ravens, Wolf-Hagen Schunck, Monika Ehrhart-Bornstein, Stefan R. Bornstein, Ingo Morano

Rationale: Adipocyte fatty acid–binding protein (FABP4) is a member of the intracellular lipid-binding protein family and is predominantly expressed in adipose tissue. Emerging evidence suggests that FABP4 plays a role in some aspects of the metabolic syndrome including the development of type 2 diabetes and atherosclerosis. We have recently reported that secretory products from human adipocytes directly and acutely depressed cardiac contractile function.

Objective: The purpose of this study was to identify this adipocyte-derived cardiodepressant factor.

Methods and Results: Through mass spectrometry and immunoblotting, we have identified this cardiodepressant factor as FABP4. FABP4 represents 1.8% to 8.1% of total protein secreted by adipocytes in extracellular medium. FABP4 acutely depressed shortening amplitude as well as intracellular systolic peak Ca^{2+} in a dose-dependent manner in isolated rat cardiomyocytes. Heart-specific FABP isoform (FABP3) revealed a similar cardiodepressant effect. The N-terminal amino acids 1 to 20 of FABP4 could be identified as the most effective cardiodepressive domain. We could exclude any effect of FABP4 on action potential duration and L-type Ca^{2+} current, suggesting a reduced excitation-contraction gain caused by FABP4 as the main inhibitory mechanism.

Conclusion: We conclude that the release of FABP4 from adipocytes may be involved in the development of cardiac contractile dysfunction of obese subjects. (*Circ Res.* 2009;105:326-334.)

Key Words: FABP4 ■ heart failure ■ adipocytes ■ metabolic syndrome

Obesity is a major risk factor in the development of the metabolic syndrome and cardiovascular diseases and seems to be directly related to heart failure independently of other risk factors.¹ Indeed, a direct relationship between elevated body mass index and increased risk for heart failure has been demonstrated, without evidence of a threshold.² Several potential mechanisms are under discussion to explain this correlation, including hemodynamic changes with cardiac overload and left ventricular remodeling and lipid accumulation into the myocardium, leading to lipoapoptosis in cardiomyocytes.³ These mechanisms, however, do not fully explain the development of heart dysfunction in obese subjects.

Adipocytes are known to produce and release a wide variety of bioactive molecules into the bloodstream.⁴ Based on these data, we have recently investigated whether secretory products from human adipocytes affect cardiac contractile function in an in vitro system of isolated rat cardiomyocytes. We have demonstrated that mature human adipocytes

release substances that strongly and acutely suppress the contraction of cardiomyocytes by attenuating intracellular Ca^{2+} levels.⁵ Our previous findings have revealed a hitherto unknown acute depressant effect of adipocyte-derived factors on cardiac contraction, suggesting a new direct role of adipose tissue in the pathogenesis of myocardium dysfunction.

Based on this initial work, we have further characterized cardiodepressant activity by fractionating adipocyte secretory products according to molecular weight and proteomic analysis, identifying adipocyte fatty acid–binding protein (FABP4) as the active agent. Fatty acid–binding proteins (FABPs) are members of a highly conserved family of cytosolic proteins with a molecular mass of 14 to 15 kDa found in different cell types, showing a high affinity for long-chain fatty acids and other hydrophobic ligands.⁶ FABP4 is predominantly expressed in adipose tissue, and accounts for $\approx 1\%$ of total cytosolic protein in human adipose tissue.⁷ Cytoplasmic FABP4 is involved in trafficking intra-

Original received July 13, 2008; resubmission received May 6, 2009; revised resubmission received July 6, 2009; accepted July 6, 2009.

From the Medical Clinic III (V.L.-Z., C.L., M.E.-B., S.R.B.), Dresden University of Technology, Germany; Institute of Cardiology (J.A.), La Habana, Cuba; Department of Pharmacology and Toxicology (T.C., U.R.), Dresden University of Technology, Germany; Max-Delbrueck-Center for Molecular Medicine (W.-H.S., I.M.), Berlin-Buch, Germany; and University Medicine Charité (I.M.), Berlin, Germany.

Correspondence to Valéria Lamounier-Zepter, Medical Clinic III, Dresden University of Technology, Fetscherstr. 74, 01307 Dresden, Germany. E-mail Valeria.Zepter@uniklinikum-dresden.de

© 2009 American Heart Association, Inc.

Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.109.200501

Non-standard Abbreviations and Acronyms

AM	adipocyte-conditioned medium
APD	action potential duration
CICR	Ca ²⁺ -induced Ca ²⁺ release
EC	excitation–contraction
FABP4	adipocyte fatty acid-binding protein
FABP3	heart fatty acid-binding protein
HBSS	Hank's balanced salts solution

cellular fatty acids and other lipid signals by interaction with functional targets.⁶ Recent evidence has supported a novel role for FABP4 in linking obesity with metabolic syndrome. Deletion of FABPs in adipocytes reduced expression of inflammatory cytokines in macrophages, and the same deletion in macrophages enhanced insulin signaling and glucose uptake in adipocytes. From these data, a potential mechanism underlying the metabolic syndrome emerges from coordinated modulation of the metabolic status in adipocytes and the inflammatory status in macrophages through pathways common to both cell types controlled by FABPs.⁸ In experimental animal models, FABP4 deficiency protects against the development of diabetes and atherosclerotic cardiovascular disease in both genetic and dietary forms of obesity.^{9,10} Humans with a functional genetic variant of the FABP4 gene, resulting in reduced adipose tissue expression of FABP4, have lower serum levels of triglycerides, and are at significantly reduced risk for type 2 diabetes and coronary artery disease.¹¹ In a recent cross-sectional study, a correlation was observed between circulating concentrations of FABP4 and metabolic syndrome.¹² Together, these clinical and experimental data support a key role for FABP4 in the development of metabolic and cardiovascular complications in obesity. In the present study, we investigated the role of FABP4 released from human adipocytes as a potential candidate linking obesity and the development of heart contractile dysfunction.

Methods

Human Adipose Tissue

Human white adipose tissue was obtained from healthy overweight and obese women (31 to 75 years old; body mass index ranging between 26 and 42 kg/m², n=15) undergoing elective surgical mammary and abdominal reduction. All women were otherwise free of metabolic and endocrine diseases. Informed consent was obtained from all donors before the surgical procedure, and the study was approved by the ethical committee of the Dresden University of Technology.

Isolation of Human Adipocytes and Preparation of Adipocyte-Conditioned Medium

Adipocytes were isolated from subcutaneous human adipose tissue as previously described.¹³ Isolated mature adipocytes were kept at 37°C in a humidified atmosphere of 5% CO₂ and cultured for 24 hours in serum-free DMEM/Nutrient Mix F12 (DMEM/F12; Life Technologies, Karlsruhe, Germany) culture medium with 100 U/mL penicillin and 100 µg/mL streptomycin. The adipocyte-conditioned medium (AM) containing all the factors released by the adipocytes was then collected and used for further experiments with isolated

adult rat cardiomyocytes. Culture medium without adipocytes was incubated in the same manner and used as control medium.

Isolation of Adult Rat Cardiomyocytes

Cardiomyocytes were isolated from male Wistar rats aged 3 months as previously described.⁵ In brief, rats were anesthetized with isoflurane followed by intraperitoneal injection of 8 µg of xylazine and 35 µg of ketamine. Hearts were rapidly removed and perfused in a Langendorff perfusion system with Ca²⁺-free Krebs–Henseleit buffer containing 10 mmol/L butanedione monoxime and 8.3 mmol/L glucose and subsequently digested by collagenase (Worthington Biochemical Corp, Lakewood, NJ). Isolated cardiomyocytes were resuspended in medium 199 supplemented with 0.2% BSA, 5% FBS, 5 mmol/L creatine, 5 mmol/L taurine, 2 mmol/L carnitine, 10 µmol/L cytosine-D-arabinofuranoside, and antibiotics. Cardiomyocytes were seeded in laminin-coated 4-well chamber slides (Nunc, Wiesbaden-Schierstein, Germany) specialized for microscopic contractility and fluorescence measurement and cultured for 4 hours in medium 199. These experiments were approved by the institutional animal care body of the Berlin federal region.

Measuring Cell Shortening and Ca²⁺ Transients

Attached cardiomyocytes were loaded with Fura-2/acetoxymethyl ester dissolved in Hank's balanced salts solution (HBSS) buffered with 10 mmol/L Hepes at pH 7.4 in the dark at room temperature for 15 minutes. Only cardiomyocytes of optically intact rod-shaped morphology with clear cross striation were analyzed. We used an Ionoptix Contractility and Fluorescence System (Ionoptix, Milton, Mass) to measure cell shortening and Ca²⁺ transients.

Incubation of Adult Rat Cardiomyocytes

Adult cardiomyocytes were electrically stimulated at 1 Hz until both shortening and Fura-2/acetoxymethyl ester signals reached a steady level. Electric pacing was then switched off, and AM or the corresponding control medium was added directly to the cardiomyocyte chamber for 5 minutes. Subsequently, the cardiomyocytes were electrically paced at 1 Hz and mechanical and fluorescence signals were collected.

The acute effect of FABP4 or the heart-specific FABP isoform (FABP3) in concentrations varying from 10 to 200 nmol/L were tested on the cardiomyocytes as described above. Both FABPs were purchased as human recombinant proteins from Cayman Chemicals (Ann Arbor, Mich).

Peptide Walking Experiments

Ten overlapping peptides (≈20 aa each) spanning the entire length of FABP4 were chemically synthesized, purified by high-performance liquid chromatography and controlled by matrix-assisted laser desorption ionization time-of-flight (Biosyntan, Berlin, Germany). All peptides were acetylated at the NH₂ terminus and ended with a COOH-terminal amide. The peptides were dissolved in dilute acetic acid at a concentration of 1 mmol/L, divided into aliquots and stored at –80°C. Peptides were diluted to final concentrations of 100 nmol/L with HBSS. The cardiodepressant activity of the FABP4 peptides was monitored as described above for FABPs.

Electrophysiological Measurements

L-type Ca²⁺ current was recorded in freshly dissociated rat cardiomyocytes using the “whole-cell” variant of the patch-clamp method at room temperature (22±2°C) as previously described.¹⁴ Action potentials were measured at 37°C with standard single-electrode current-clamp technique (Axopatch 200, Axon Instruments, Foster City, Calif).

Detailed description of electrophysiological measurements is available in the Online Data Supplement at <http://circres.ahajournals.org>.

Mass Spectrometry

AM from 6 different preparations were pooled and concentrated 16-fold to a protein concentration of 372.3 µg/mL on a centrifuge

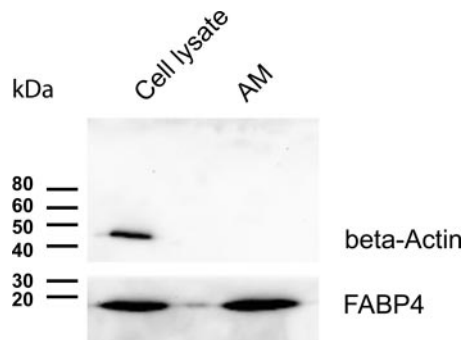


Figure 1. Western blot analysis of lysate from human adipocytes and AM. FABP4 is abundantly present inside adipocytes as well as in the AM. Protein (1 μ g) from cell lysates or AM was separated by 12% SDS-PAGE and immunoblotted with a polyclonal anti- β -actin or with a polyclonal anti-FABP4 antibody.

cartridge (Centricon, Millipore GmbH, Schwalbach, Germany) with a cutoff of 1 kDa. Protein (9.0 μ g) was separated on 10% polyacrylamide gel (Invitrogen GmbH, Karlsruhe, Germany) under reducing conditions. Proteins were identified at the Mass Spectrometry Facility (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Protein bands were visualized by staining with Coomassie; the full lane was cut into 17 slices, whereas visible bands were always sliced separately. Excised gel plugs were cut into approximate 1 \times 1 \times 1 mm cubes, in-gel digested with trypsin, and extracted from gel matrix with acetonitrile and 5% formic acid.¹⁵ Recovered peptides were subjected to liquid chromatography/tandem mass spectrometric analysis on LTQ ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) under conditions described by Waridel et al.¹⁶ and acquired tandem mass spectra were searched with reference to an MSDB database using MASCOT version 2.1 software (Matrix Science Ltd, London, UK) installed on a local server. Protein hits were considered confident where at least 2 tandem mass spectra, each of which matched the corresponding database sequences with peptide ion scores, exceeded the confidence threshold suggested by MASCOT ($P < 0.05$).

Secreted proteins with a canonical N-terminal signal peptide were identified by SignalP 3.0.¹⁷ Prediction of noncanonical, that is, non-signal peptide-triggered protein secretion was performed by SecretomeP 2.0.¹⁸ Proteins with a signal peptide predicted by SignalP were considered as secreted proteins via canonical pathway (endoplasmic reticulum/Golgi-dependent pathway). If no signal peptide was predicted but the neural network score exceeded a value of 0.6, proteins were classified as secreted via the noncanonical pathway.

Microvesicle Preparation From Conditioned Media

Microvesicles were prepared from AM as previously described.¹⁹ Briefly, conditioned medium was first centrifuged at 1000g for 5 minutes and then at 15 000g for 15 minutes to remove cell debris and aggregates. The supernatant was ultracentrifuged at 100 000g for 1 hour. Pelleted vesicles were resuspended in phosphate-buffered saline and ultracentrifuged again for washing at 100 000g for 15 minutes. The pelleted vesicles and supernatant obtained were then subjected directly to Western blotting or resuspended in HBSS solution in the same concentration as the original volume and tested on the cardiomyocytes.

Measurement of FABP4 and Total Protein Concentration in AM

FABP4 concentrations in AM were determined by ELISA for human FABP4 (BioVendor Laboratory Medicine Inc, Modrice, Czech Republic) according to the instructions of the manufacture. Protein concentrations were quantified by using a Bradford protein assay kit (Bio-Rad, Munich, Germany).

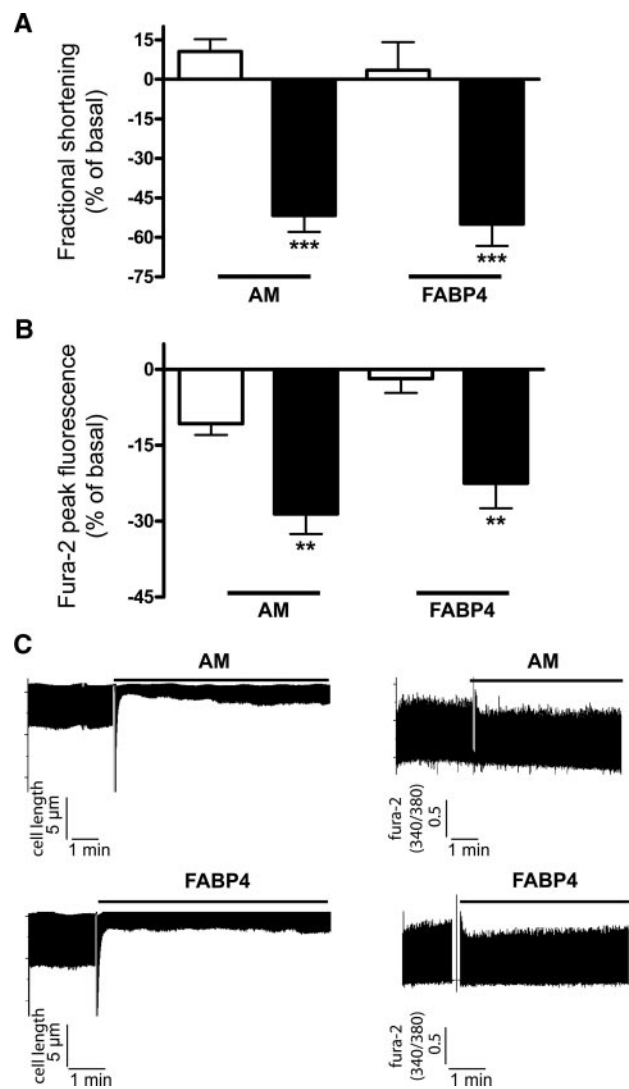


Figure 2. Effect of AM and FABP4 on fractional shortening (A) and Fura-2 peak fluorescence (B) on adult rat cardiomyocytes. Left, Control medium (open bars) and AM (filled bars). Right, Control vehicle for FABP4 (open bars) and FABP4 (filled bars). AM and respective control were used at a dilution of 1:6. FABP4 was used in a concentration of 100 nmol/L. Values are expressed as percentage change in shortening amplitude and Fura-2 signals during the preincubation period. Both AM and FABP4 significantly decreased fractional shortening (A) and Fura-2 peak fluorescence (B); mean \pm SEM; $n = 7$ to 10 (control) or 11 to 13 (AM and FABP4). ** $P < 0.01$; *** $P < 0.001$. C, Original chart recording of cardiomyocyte shortening amplitudes (left) and Fura-2 peak fluorescence (right) before and after incubation with AM (top) or FABP4 (bottom).

Cell Lysis and Immunoblotting Analysis

Adipocytes were lysed with lysis buffer containing (mmol/L): 20 Hepes (pH 7.9), 350 NaCl, 20% Glycerol, 1 MgCl₂, 0.5 EDTA, 0.1 EGTA, 1% Tergitol, 4% protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany), 1% phosphatase inhibitor cocktail (Sigma-Aldrich, Munich, Germany).

Cell lysates, AM, microvesicle fraction of adipocyte medium, and 0.25 μ g of FABP4 for comparison were loaded onto a 12% SDS-polyacrylamide gel and electrically transferred to a nitrocellulose membrane (Invitrogen GmbH, Karlsruhe, Germany) according to standard procedures. The blots were incubated with an affinity-purified specific FABP4 polyclonal antibody (Cayman Chemical) as the first antibody at a concentration of 1 μ g/mL at 4°C overnight or

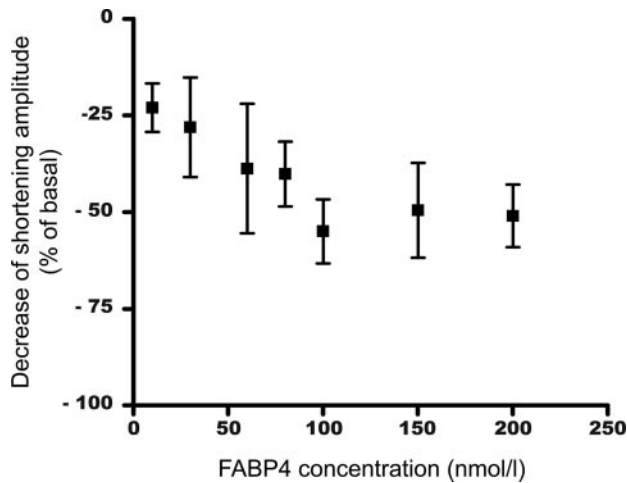


Figure 3. Concentration dependence of negative inotropic activity of FABP4. Cardiomyocytes were incubated with FABP4 in concentrations between 10 and 200 nmol/L. Maximal effect on shortening amplitude was reached in a concentration of 100 nmol/L; $n=4$ to 7 experiments for each concentration.

with a specific polyclonal antibody against β -actin (Cell Signaling Technology, Danvers, Mass) for control, and subsequently with a goat anti-rabbit–horseradish peroxidase conjugate (Bio-Rad, Munich, Germany). Immunodetection was performed with an enhanced chemiluminescence detection kit (Pierce SuperSignal Kit, Pierce, Bonn, Germany), and the protein bands were analyzed using SynGene Gene Tools (SynGene, Cambridge, UK).

Statistics

Experiments were performed on at least 3 individual heart preparations. Six to 8 cardiomyocytes were used for functional analysis in each heart preparation. We used unpaired Student's t test for significance analysis. Values are expressed as their means \pm SEM.

Results

Human Adipocytes Release FABP4 Into Extracellular Medium

Mass spectrometry–based fingerprinting identified 386 proteins in conditioned medium from isolated human adipocytes. Eighty of these proteins were proteins with typical signaling sequences including previously described adipokines, such as adiponectin and complement factor 3, or constituents of extracellular matrix, such as laminin or collagen (data not shown). Interestingly, human FABP4 was identified as the major component in the ≈ 15 -kDa band. Noncanonical secretion of FABP4 is predicted by Secretome 2.0 Server (neural network score, 0.765).

Immunoblotting analysis revealed an abundance of FABP4 in cell lysates from human adipocytes and in AM. In contrast, β -actin, a cytoskeleton protein, was detected in the adipocyte lysates but not in conditioned medium from adipocytes, indicating that FABP4 in the conditioned medium was not released from adipocytes by nonspecific cell lysis (Figure 1).

We next measured the concentrations of FABP4 in conditioned media from adipocytes obtained from different individuals using a commercially available sandwich ELISA assay. This assay has previously been shown to be highly specific for human FABP4, with no detectable cross-reactivity with other types of FABPs.¹² Adipocytes in our cell

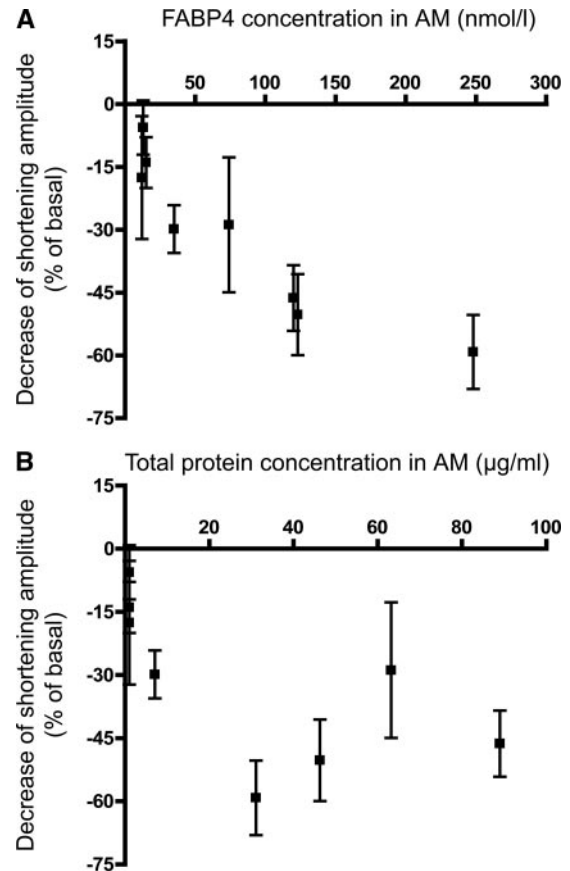


Figure 4. A, Relationship between negative inotropic activity on cardiomyocyte contraction of AM from different adipocytes preparations and concentrations of FABP4 found in each AM. B, Negative inotropic activity of eight adipocyte-media was normalized to total protein concentration found in each media, showing no correlation between total protein concentration and negative inotropic activity of media; $n=4$ to 6 different experiments for each AM.

culture system released high amounts of FABP4, resulting in concentrations between 12 and 250 nmol/L in conditioned medium. FABP4 represented 1.8% to 8.1% of the total protein in AM ($3.89 \pm 0.78\%$; mean \pm SEM).

FABP4 Acutely Inhibits Cardiomyocyte Contraction

We tested a possible direct effect of FABP4 on cardiomyocyte contraction using similar concentrations as released by human adipocytes in our cell culture model. At a concentration of 100 nmol/L, FABP4 significantly decreased both the shortening amplitude from 5.4 ± 0.8 to 2.6 ± 0.7 μm , that is $-55.0 \pm 8.3\%$ ($P < 0.001$), and Fura-2 peak fluorescence signal by $-22.5 \pm 4.9\%$ ($P < 0.01$; Figure 2). On incubation with the vehicle buffer, a low, nonsignificant decrease in Fura-2 peak fluorescence amounting to $-2.0 \pm 2.8\%$ was observed. Thus, incubation with FABP4 induced a net decrease of approximately 20% in Fura-2 peak fluorescence.

Similarly, incubation with AM generated a significant decline in both shortening amplitude from 6.0 ± 0.6 to 2.9 ± 0.6 μm , that is $-51.5 \pm 6.2\%$ ($P < 0.001$), and Fura-2 peak fluorescence signal by $-28.6 \pm 3.9\%$ ($P < 0.01$). As previously described,⁵ a minor attenuation of Fura-2 peak

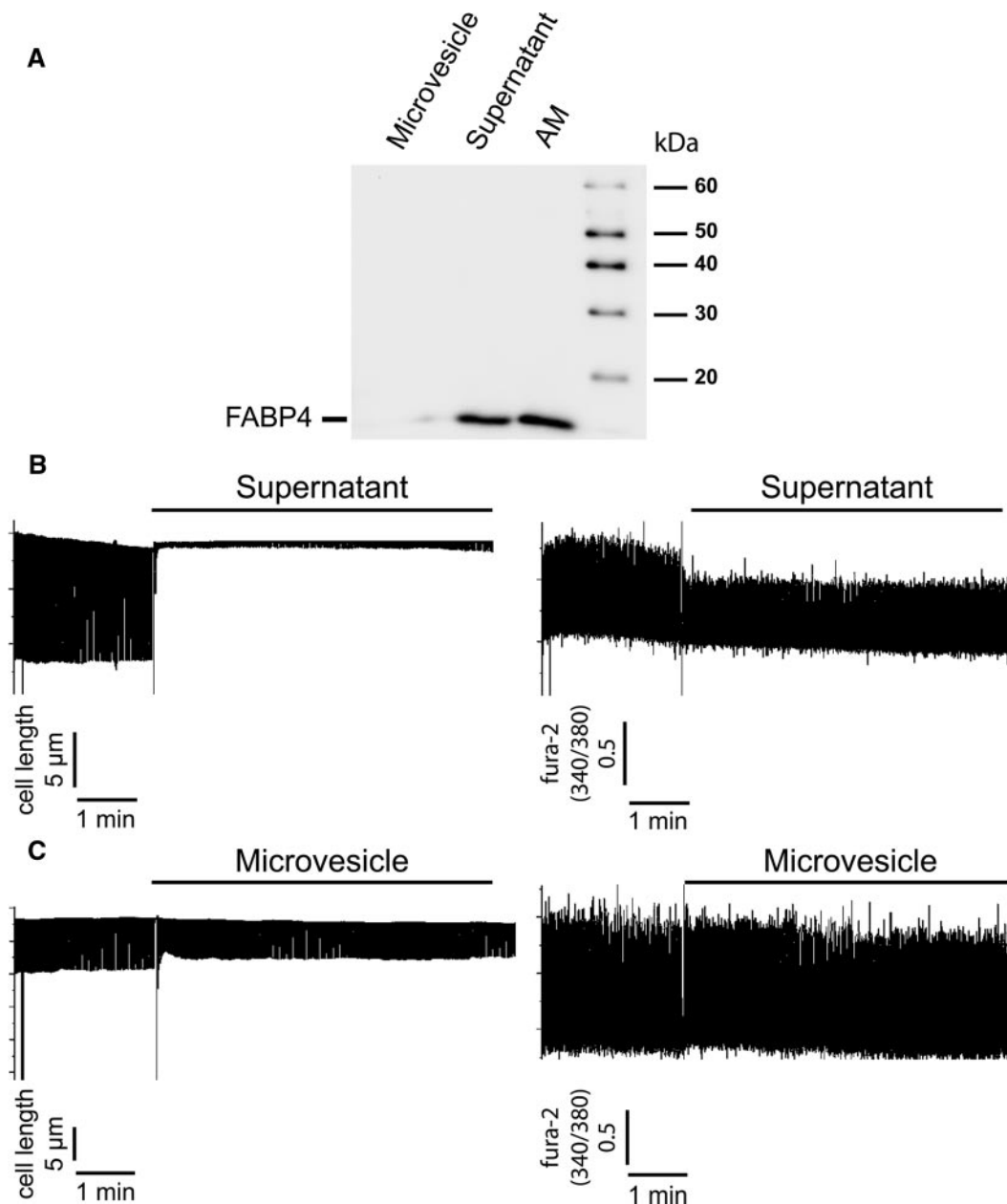


Figure 5. A, Immunoblotting of FABP4 in AM. AM was fractionated into microvesicle and supernatant by ultracentrifugation. Aliquots of microvesicle fraction, supernatant and unfractionated AM were separated by SDS-PAGE followed by immunoblotting with an anti-FABP4 antibody. FABP4 was predominantly found in the supernatant fraction, representing 86% of total amount in unfractionated AM. B and C, Original chart recording of cardiomyocyte shortening amplitudes (left) and Fura-2 peak fluorescence (right) before and after incubation with supernatant (B) and microvesicle (C) fractions of AM. Inotropic negative effect of AM was mainly observed in the supernatant fraction.

fluorescence at $-10.8 \pm 2.2\%$ was observed on incubation with control medium (Figure 2). Thus, the net decline in Fura-2 peak fluorescence after incubation with AM was $\approx 18\%$, similar to the FABP4-induced net decline in Fura-2 peak fluorescence.

The inotropic negative effect of FABP4 was concentration-dependent at a maximal effective concentration of 100 nmol/L and an EC_{50} of 19.2 nmol/L (Figure 3).

The diastolic length of cardiomyocytes remained almost constant in cells treated with either FABP4 (113.6 ± 4.1 μm before and 113.7 ± 4.1 μm after incubation) or AM (103.2 ± 4.4 μm before and 103.7 ± 4.5 μm after incubation).

No morphological changes were observed after incubation with FABP4 or AM.

FABP4 Concentrations in Adipocyte Medium Are Positively Correlated With Cardiodepressant Activity

We then investigated the relationship between the amount of FABP4 released from the adipocytes into extracellular medium and the inotropic negative activity of this AM. The cardiodepressant activity of varying AM as represented by the inhibition of shortening amplitude in cardiomyocytes was directly correlated to the concentrations of FABP4 found in

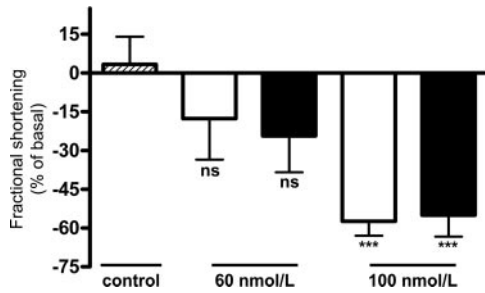


Figure 6. Effect of FABP3 and FABP4 on fractional shortening on cardiomyocytes. Left, Medium with solvent as control. Right, FABP3 (open bars) and FABP4 (filled bars) in concentrations of 60 and 100 nmol/L. Both FABP3 and FABP4 significantly decreased fractional shortening in a concentration of 100 nmol/L in comparison to control. The effects of FABP3 and FABP4 did not differ between each other in both concentrations used. Mean \pm SEM; $n=7$ (control), 5 to 8 (FABP3) or 6 to 11 (FABP4); ns indicates not significant. *** $P<0.001$.

varying AM (Figure 4A). AM showed a maximal cardiodepressant activity at a concentration of 200 nmol/L FABP4 and an EC_{50} of 35 nmol/L. In contrast, no correlation was observed between cardiodepressant activity and total protein content in AM (Figure 4B), suggesting that there is no unspecific correlation.

Low FABP4 in Microvesicles Correlate With Weak Cardiodepression

Microvesicles are vesicles released from the plasma membrane of various cell types containing cell surface proteins and some cytoplasmic components. Murine adipocyte 3T3-L1 cells were recently reported to secrete microvesicles containing a variety of secreted, integral, and cytosolic proteins.¹⁹ To analyze whether FABP4 is released by adipocytes via this mechanism, microvesicle fractions of AM were prepared by ultracentrifugation and further assessed by immunoblotting with specific anti-FABP4 antibody (Figure 5A). FABP4 was mainly recovered in the supernatant fraction (representing 86% of total amount in unfractionated adipocyte medium). The microvesicle fraction contained only 8% of total amount of FABP4. Accordingly, cardiodepressant activity was mainly observed in the supernatant fraction ($60.9 \pm 10.9\%$ decline in shortening amplitude, and $31.7 \pm 4.9\%$ decline in Fura-2 peak fluorescence, $n=7$, $P<0.001$; Figure 5B), whereas the microvesicle fraction had a minor cardiodepressant effect ($28.1 \pm 9.5\%$ decline in shortening amplitude, and $23.9 \pm 4.2\%$ decline in Fura-2 peak fluorescence, $n=7$, $P<0.01$; Figure 5C).

FABP3 and FABP4 Revealed Similar Cardiodepressant Effects

In a separate set of experiments, we investigated a possible cardiodepressant activity of FABP3. FABP3 showed a similar depressive effect on cardiomyocytes contraction as FABP4 (Figure 6).

FABP4 Did Not Modulate L-Type Ca^{2+} Channel Activity or Action Potential Duration

We investigated the current–voltage curves of L-type Ca^{2+} channels under basal conditions and during FABP4 (150

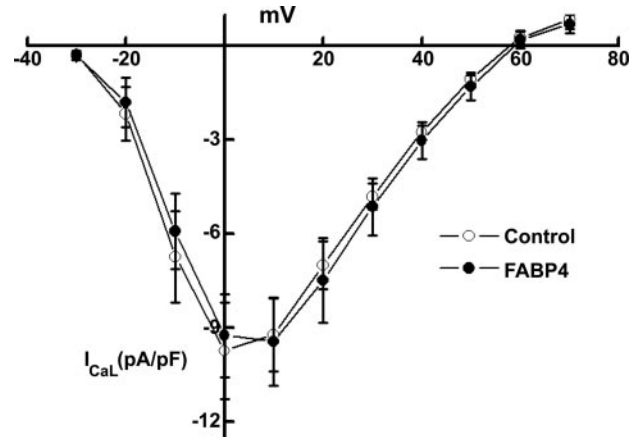


Figure 7. Current–voltage relationship of L-type Ca^{2+} current in pA/pF vs mV of cardiomyocytes before (control, open symbols) and after FABP4 (150 nmol/L) exposure (closed symbols). Mean \pm SEM; $n=14$ (control) or 21 (FABP4).

nmol/L) stimulation of rat cardiomyocytes. No difference of basal L-type Ca^{2+} current between both groups was detected (Figure 7).

Under control conditions, resting membrane potential in cardiomyocytes was -75.4 ± 2.0 mV ($n=5$) and remained unchanged during exposure to 150 nmol/L FABP4. Action potential duration (APD) declined after 5 minutes from 53.6 ± 8.1 to 46.8 ± 7.4 ms (APD_{90}) and from 16.2 ± 3.6 to 14.1 ± 3.1 ms (APD_{20} ; $P<0.05$ for both parameters, $n=5$). However, this time-dependent decline was not significantly different from the decline observed in time-matched FABP4-treated cardiomyocytes.

The N Terminus of FABP4 Confers the Cardiodepressant Effect

Cardiomyocytes were incubated with synthetic peptides (100 nmol/L each) that overlap and cover the complete FABP4 molecule. Remarkably, peptides could be grouped into 3 clusters, namely, peptides with pronounced inhibitory action (cluster a), peptides with more stimulating effect (cluster b), and peptides with a smaller or no inhibitory activity (cluster c) (Figure 8). The N terminus expressed more cluster a, whereas clusters b and c distributed along the C terminus. The most prominent inhibitory activity could be observed with the N-terminal peptide amino acids 1 to 20 (mcdafvgtwklvssenfdd), which revealed an approximately 40% inhibition of cardiomyocyte contraction. A second minor inhibitory activity was observed with the peptide amino acids 46 to 59 (ngdvitkxsestfk), which revealed around 18% inhibition of cardiomyocyte contraction.

Discussion

We previously suggested a new pathophysiological mechanism of cardiac dysfunction in obesity caused by the release of cardiodepressant substances from adipocytes.⁵ In the present study, we have now identified this adipocyte-dependent cardiodepressant factor as the FABP4. Adipocytes are known to produce FABP4, and are thought to be a major source of FABP4 in the circulation of patients with the metabolic syndrome.¹² The molecular size of FABP4 (15

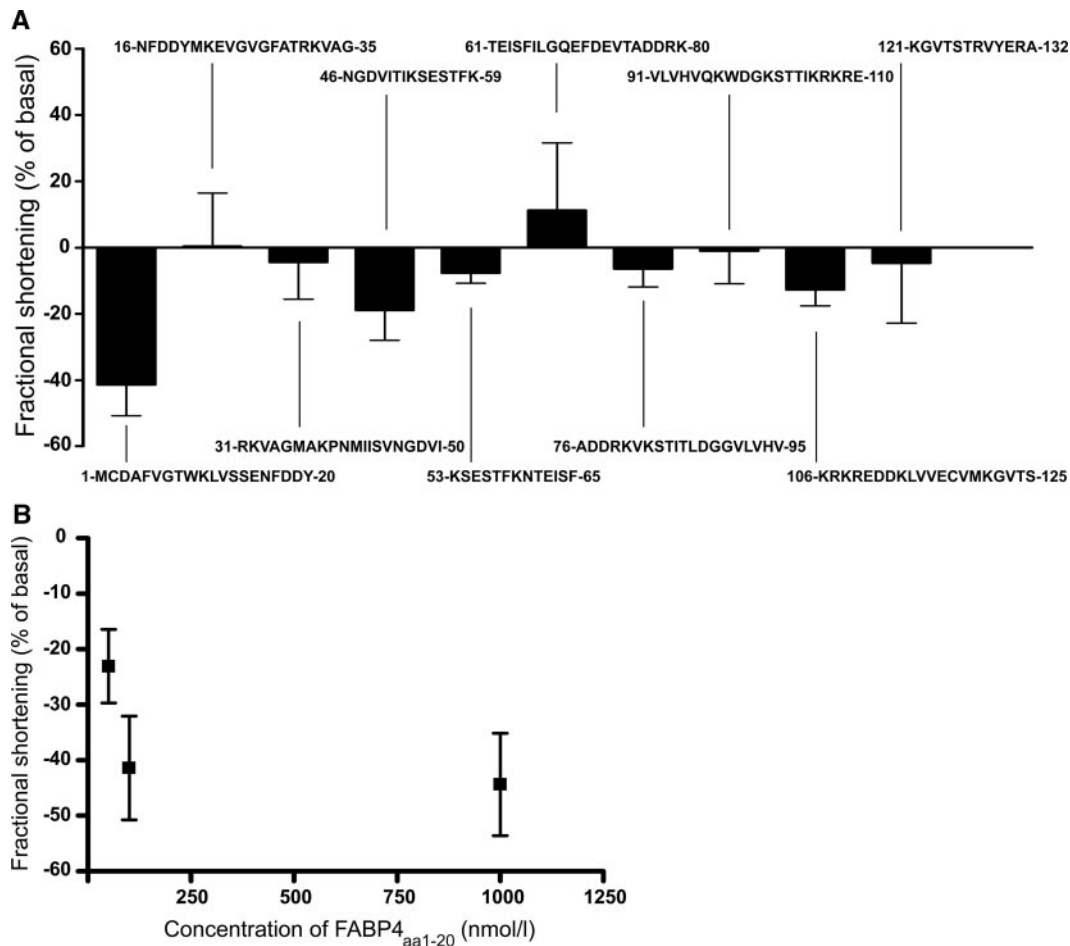


Figure 8. A, Analysis of the cardiodepressant domain of FABP4 by peptide walking. Synthetic FABP4 peptides (individual sequence at the top and bottom) were monitored for effect on fractional shortening in cardiomyocytes. Data are means \pm SEM; $n=6$ to 8 experiments for each peptide. B, Concentration dependence of cardiodepressant effect of FABP4 peptide 1 to 20 (MCDAFVGWTWKLVSSENFDDY). Cardiomyocytes were incubated with FABP4 peptide 1 to 20 in concentrations 50, 100, and 1000 nmol/L. Maximal response was reached with 100 nmol/L; $n=7$ to 11 experiments for each concentration.

kDa) agrees with our previous size exclusion experiments, which indicated a molecular weight of between 10 and 30 kDa.⁵ Human adipocytes in our primary cell culture system released high amounts of FABP4 into the extracellular medium. FABP4 at similar concentrations to those released by the adipocytes acutely inhibited cardiomyocyte contraction. This effect was concentration-dependent and, as with the previously described adipocyte-cardiodepressant factor, reduced intracellular Ca^{2+} -transient. Furthermore, the cardiodepressant effect of the individual AM correlated with the FABP4 concentration present in the corresponding medium. In support of the specificity of this correlation, no correlation exists between total protein concentration and the cardiodepressant effect of AM.

Recent studies from animal models support a novel role for FABP4 in linking obesity with many features of the metabolic syndrome. Mice lacking FABP4 exhibit a protective phenotype against the development of insulin resistance associated with genetic or diet-induced obesity.^{9,10} FABP4 is also expressed in macrophages, which seem to be a critical site of action for FABP4 in the development of atherosclerosis^{20,21} and inflammation.⁸ Indeed, total or macrophage-

specific deficiency of FABP4 leads to significant protection against severe atherosclerosis in apolipoprotein E-deficient mice.^{20,22} More recently, the use of an inhibitor of FABP4 that competitively inhibits the binding of endogenous fatty acids was effective against severe atherosclerosis and type 2 diabetes in a mouse model of atherosclerosis and obesity.²³ Consistent with these animal studies, a close positive correlation between circulating concentrations of FABP4 and features of the metabolic syndrome has been revealed in humans.¹² Based on observations that FABP4 positively correlates with body mass index and fat percentage, and that the murine preadipocyte cell-line 3T3-L1 releases FABP4 into the extracellular medium, adipose tissue has been suggested as being the main source of circulating FABP4.¹² Our findings that human adipocytes in primary cell culture also release FABP4 into the extracellular medium support the role of adipose tissue in the secretion of FABP4 into circulation.

Although several studies have pointed to a role of FABP4 in the development of some features of the metabolic syndrome, the pathophysiological mechanisms of circulating FABP4 in mediating the metabolic and cardiovascular complications of obesity remain undetermined. Cytoplasmic

FABP4 is believed to intercede the intracellular trafficking and targeting of fatty acids.⁶ Circulating FABP4 may participate in transporting free fatty acids or other lipid hormones in the bloodstream, which may in turn mediate the metabolic features of obesity. These are the first research findings to suggest a direct bioactive role of FABP4 in heart function, independently of its role as a transport protein. Interestingly, Vural et al²⁴ have recently reported FABP4 expression in epicardial adipose tissue, with elevated levels in patients with metabolic syndrome. Epicardial fat tissue accumulates around the heart and is directly related to intraabdominal visceral fat and other features of the metabolic syndrome.²⁵ There are now compelling data pointing to a role of epicardial fat tissue in modulating heart morphology and function.²⁶ The absence of a fibrous fascial layer between epicardial fat tissue and underlying myocardium permits a close anatomic relationship between both tissues, thus paving the way for factors released from adipocytes to influence myocardial function directly.²⁷ Consequently, the FABP4 expression found in epicardial fat tissue supports the hypothesis of a direct paracrine effect exerted by FABP4 on heart dysfunction development in patients with obesity and metabolic syndrome. Additionally, the increase in circulating FABP4 released from subcutaneous and/or visceral adipose tissue in obese subjects could mediate heart dysfunction in those subjects.

The precise molecular mechanism of the cardiodepressive effect of FABP4 is not yet understood. In cardiomyocytes, Ca^{2+} -induced Ca^{2+} release (CICR) links excitation to contraction; eg, Ca^{2+} crossing the cell membrane triggers the release of a larger quantity of Ca^{2+} from the sarcoplasmic reticulum. The primary trigger for CICR is L-type Ca^{2+} current. In experimental investigation of CICR, a measure frequently derived is excitation–contraction (EC) coupling “gain,” defined as the ratio of the peak sarcoplasmic reticulum Ca^{2+} release flux to the peak of Ca^{2+} flux through the L-type Ca^{2+} channels. Hence, EC coupling gain quantifies the amplification provided by CICR.²⁸ In our experiments, FABP4 reduced intracellular Ca^{2+} transient, but an effect on APD and L-type Ca^{2+} current could be excluded, thus suggesting a reduced EC coupling gain caused by FABP4. Interestingly, a defective EC gain has already been observed in heart failure.^{29,30}

Peptide walking of FABP4 identified the N-terminal FABP4 fragment amino acids 1 to 20, which comprises the β A-sheet and half of α 1-helix, as the main cardiodepressant domain, whereas peptides from the C-terminal domain are less inhibitory. These data confirm that FABP4 itself, rather than an unidentified molecule associated with FABP4, confers the cardiodepressive activity.

The heart-specific fatty acid-binding protein FABP3 revealed a similar cardiodepressive effect. Sequence comparison of the N termini amino acids 1 to 20 between both FABPs (FABP4, accession no. NP_001433.1; FABP3, accession no. NP_004093.1) revealed 80% identity (NCBI Blast), whereas the C termini of FABP3 and FABP4 are not homologous. In addition, crystal structures of the N termini of FABP3 (Protein Data Bank code 1g5w) and FABP4 (Protein Data Bank code 2hnx) show a common characteristic β -sheet/

helix–loop–helix motif, which provide structural evidence for their similar inhibitory effect on cardiomyocytes.

Both FABPs are effective in the extracellular medium, thus suggesting a surface receptor for heart and adipocyte FABP. This idea is supported in several ways: labeled FABP4 cannot penetrate living adult cardiomyocytes (data not shown), excluding an intracellular mechanism of cardiodepression by FABP4. Furthermore, the heart FABP isoform selectively binds to a high affinity plasma membrane receptor on cardiomyocytes.³¹ According to their common inhibitory activity and structure of their functional domains, it may be expected that FABP4 also binds to a specific surface receptor on cardiomyocytes to inhibit cardiomyocyte function.

In conclusion, our data show that FABP4 is released from human adipocytes and elicits a direct and acute Ca^{2+} -dependent suppressing effect on cardiomyocyte contraction. The elevated levels of circulating FABP4 and/or locally expressed FABP4 in epicardial fat tissue as observed in obese subjects may be partially responsible for the development of heart dysfunction in these subjects.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Uta Buro. We thank Dr M. Weiße-Lögering (Pirna Hospital) and her team for the help in obtaining human adipose tissue and Kathleen Eisenhofer for proofreading of the manuscript.

Sources of Funding

This study was supported by the “Kompetenznetz Adipositas” (Competence Network for Obesity), funded by the German Federal Ministry of Education and Research (grant 01G10833), and by MeDDrive-33, Dresden University of Technology (to V.L.Z.); and by German Research Foundation grant EH161/4-1 (to M.E.B. and S.R.B.).

Disclosures

None.

References

1. He J, Ogden LG, Bazzano LA, Vupputuri S, Loria C, Whelton PK. Risk factors for congestive heart failure in US men and women: NHANES I epidemiologic follow-up study. *Arch Intern Med*. 2001;161:996–1002.
2. Kenchaiah S, Evans JC, Levy D, Wilson PW, Benjamin EJ, Larson MG, Kannel WB, Vasan RS. Obesity and the risk of heart failure. *N Engl J Med*. 2002;347:305–313.
3. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation*. 2006;113:898–918.
4. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*. 2004;89:2548–2556.
5. Lamounier-Zepter V, Ehrhart-Bornstein M, Karczewski P, Haase H, Bornstein SR, Morano I. Human adipocytes attenuate cardiomyocyte contraction: characterization of an adipocyte-derived negative inotropic activity. *FASEB J*. 2006;20:1653–1659.
6. Hertz AV, Bernlohr DA. The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrinol Metab*. 2000;11:175–180.
7. Baxa CA, Sha RS, Buelt MK, Smith AJ, Matarese V, Chinander LL, Boundy KL, Bernlohr DA. Human adipocyte lipid-binding protein: purification of the protein and cloning of its complementary DNA. *Biochemistry*. 1989;28:8683–8690.
8. Furuhashi M, Fucho R, Görgün CZ, Tuncman G, Cao H, Hotamisligil GS. Adipocyte/macrophage fatty acid-binding proteins contribute to metabol-

- ic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest*. 2008;118:2640–2650.
9. 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.
 10. Uysal KT, Scheja L, Wiesbrock SM, Bonner-Weir S, Hotamisligil GS. Improved glucose and lipid metabolism in genetically obese mice lacking aP2. *Endocrinology*. 2000;141:3388–3396.
 11. Tuncman G, Erbay E, Hom X, De Vivo I, Campos H, Rimm EB, Hotamisligil GS. A genetic variant at the fatty acid-binding protein aP2 locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease. *Proc Natl Acad Sci U S A*. 2006;103:6970–6975.
 12. Xu A, Wang Y, Xu JY, Stejskal D, Tam S, Zhang J, Wat NM, Wong WK, Lam KS. Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. *Clin Chem*. 2006;52:405–413.
 13. Ehrhart-Bornstein M, Lamounier-Zepter V, Schraven A, Langenbach J, Willenberg HS, Barthel A, Hauner H, McCann SM, Scherbaum WA, Bornstein SR. Human adipocytes secrete mineralocorticoid-releasing factors. *Proc Natl Acad Sci U S A*. 2003;100:14211–14216.
 14. Alvarez J, Hamplova J, Hohaus A, Morano I, Haase H, Vassort G. Calcium current in rat cardiomyocytes is modulated by the carboxyl-terminal ahnak domain. *J Biol Chem*. 2004;279:12456–12461.
 15. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc*. 2006;1:2856–2860.
 16. Waridel P, Frank A, Thomas H, Surendranath V, Sunyaev S, Pevzner P, Shevchenko A. Sequence similarity-driven proteomics in organisms with unknown genomes by LC-MS/MS and automated de novo sequencing. *Proteomics*. 2007;7:2318–2329.
 17. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol*. 2004;340:783–795.
 18. Bendtsen JD, Jensen LJ, Blom N, von Heijne G, Brunak S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel*. 2004;17:349–356.
 19. Aoki N, Jin-no S, Nakagawa Y, Asai N, Arakawa E, Tamura N, Tamura T, Matsuda T. Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles. *Endocrinology*. 2007;148:3850–3862.
 20. Makowski L, Hotamisligil GS. The role of fatty acid binding proteins in metabolic syndrome and atherosclerosis. *Curr Opin Lipidol*. 2005;16:543–548.
 21. Makowski L, Boord JB, Maeda K, Babaev VR, Uysal KT, Morgan MA, Parker RA, Suttles J, Fazio S, Hotamisligil GS, Linton MF. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med*. 2001;7:699–705.
 22. Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, Hotamisligil GS. Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice. *Circulation*. 2004;110:1492–1498.
 23. Furuhashi M, Tuncman G, Gorgun CZ, Makowski L, Atsumi G, Vailancourt E, Kono K, Babaev VR, Fazio S, Linton MF, Sulsky R, Robl JA, Parker RA, Hotamisligil GS. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature*. 2007;447:959–965.
 24. Vural B, Atalar F, Ciftci C, Demirkan A, Susleyici-Duman B, Gunay D, Akpinar B, Sagbas E, Ozbek U, Buyukdevrim AS. Presence of fatty-acid-binding protein 4 expression in human epicardial adipose tissue in metabolic syndrome. *Cardiovasc Pathol*. 2008;17:392–398.
 25. Iacobellis G, Leonetti F. Epicardial adipose tissue and insulin resistance in obese subjects. *J Clin Endocrinol Metab*. 2005;90:6300–6302.
 26. Iacobellis G, Sharma AM. Epicardial adipose tissue as new cardio-metabolic risk marker and potential therapeutic target in the metabolic syndrome. *Curr Pharm Des*. 2007;13:2180–2184.
 27. Sacks HS, Fain JN. Human epicardial adipose tissue: a review. *Am Heart J*. 2007;153:907–917.
 28. Wier WG. Gain and cardiac EC coupling. Revisited and revised. *Circ Res*. 2007;101:533–535.
 29. Gómez AM, Guatimosim S, Dilly KW, Vassort G, Lederer WJ. Heart failure after myocardial infarction: altered excitation-contraction coupling. *Circulation*. 2001;104:688–693.
 30. Gómez AM, Valdivia HH, Cheng H, Lederer MR, Santana LF, Cannell MB, McCune SA, Altschuld RA, Lederer WJ. Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. *Science*. 1997;276:800–806.
 31. Burton PB, Hogben CE, Joannou CL, Clark AG, Hsuan JJ, and Totty NF, Sorensen C, Evans RW, Tynan MJ. Heart fatty acid binding protein is a novel regulator of cardiac myocyte hypertrophy. *Biochem Biophys Res Commun*. 1994;205:1822–1828.