Molecules and Cells

Endophilin A2: A Potential Link to Adiposity and Beyond

Assim A. Alfadda[1,2,*], Reem M. Sallam[1,3], Rukhsana Gul[1], Injae Hwang[4], and Sojeong Ka[5]

[1]Obesity Research Center, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia, [2]Department of Medicine, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia, [3]Clinical Chemistry Unit, Pathology Department, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia, [4]Department of Biological Sciences, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 08826, Korea, [5]Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea
*Correspondence: aalfadda@ksu.edu.sa
http://dx.doi.org/10.14348/molcells.2017.0137
www.molcells.org

Adipose tissue plays a central role in regulating dynamic crosstalk between tissues and organs. A detailed description of molecules that are differentially expressed upon changes in adipose tissue mass is expected to increase our understanding of the molecular mechanisms that underlie obesity and related metabolic co-morbidities. Our previous studies suggest a possible link between endophilins (SH3Grb2 proteins) and changes in body weight. To explore this further, we sought to assess the distribution of endophilin A2 (EA2) in human adipose tissue and experimental animals. Human paired adipose tissue samples (subcutaneous and visceral) were collected from subjects undergoing elective abdominal surgery and abdominal liposuction. We observed elevated EA2 gene expression in the subcutaneous compared to that in the visceral human adipose tissue. EA2 gene expression negatively correlated with adiponectin and chemerin in visceral adipose tissue, and positively correlated with TNF-α in subcutaneous adipose tissue. EA2 gene expression was significantly downregulated during differentiation of preadipocytes *in vitro*. In conclusion, this study provides a description of EA2 distribution and emphasizes a need to study the roles of this protein during the progression of obesity.

Keywords: adipose tissue, endophilin A2, obesity, SH3GL1

INTRODUCTION

Obesity is one of the most serious threats to humans worldwide, and its pathogenesis is a complex combination of genetic predisposition, lifestyle, and environmental factors (O'Rahilly, 2016). Adipose tissue is an active endocrine organ that is involved in a number of metabolic activities and plays a critical role in processes, such as energy homeostasis, cytokine production, inflammation, and immunity (McGown et al., 2014). It is the source of several active mediators, hormones, and signaling molecules, collectively termed "adipokines" (Cao, 2014). Similar to other chronic diseases, such as diabetes mellitus (DM), atherosclerosis, dyslipidemia and cardiovascular diseases (CVD), obesity has been associated with a state of systemic low-grade chronic inflammation (Hotamisligil, 2006). The molecular mechanisms underlying this state are not yet fully understood. Nevertheless, strong evidence is accumulating to support mutual crosstalk between adipocytes and cells within the stromovascular fraction (SVF) of adipose tissue, specifically to macrophages (Suganami et al., 2005; Sun et al., 2011; Wellen and Hotamisligil, 2005). Moreover, adipose tissue actively interacts with more distant metabolically critical tissues and organs, such as the skeletal muscle, liver, brain, and the cardiovascular

Received 19 July, 2017; revised 7 September, 2017; accepted 20 September, 2017; published online 6 November, 2017

eISSN: 0219-1032
© The Korean Society for Molecular and Cellular Biology. All rights reserved.
This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/.

system (Romacho et al., 2014). Adipokines provide the means to establish and maintain this complex, yet tightly regulated integration and dynamic crosstalk (Speakman and Mitchell, 2011).

Based on the central role played by adipokines, their study remains an area of active research. This includes both the discovery of novel molecules and the recognition of adipose tissue as a source of cytokines; hence the reason for adding them to the list of known adipokines. Owing to the active, pleotropic, and often interconnected metabolic roles of adipokines, more research in this field promises a better understanding of the pathogenesis of obesity and associated comorbidities. We (Alfadda et al., 2014) and others (Bouwman et al., 2009; Gu et al., 2013) have studied the proteomic profiles of biological fluids and adipose tissue obtained from obese subjects before and after various weight loss strategies. We expect that an in-depth analysis of molecules that are differentially expressed in individuals with different body weights, or upon significant weight loss, will increase our understanding of the pathophysiology of obesity and identify potential target(s) for the prevention and treatment of this serious epidemic. However, a crucial initial step in this process is a more detailed description of such potential mediators.

One protein that was significantly elevated in the urine of subjects who underwent weight reduction as compared to its levels in the same subjects before weight loss was Src-homology 3 (SH3) domain-binding glutamic acid-rich-like protein 3 (Alfadda et al., 2014). We investigated whether this was accompanied by differential expression of SH3-domain containing proteins, and selected the endophilins family (SH3Grb2 proteins) for further study. Endophilin A2 (EA2), also called SH3-domain GRB2-like 1 or SH3GL1, was chosen owing to its ubiquitous tissue distribution, compared to other family members that show more tissue/organ-specific expression (Kjaerulff et al., 2011). Endophilins, in association with other proteins and lipids, are involved in synaptic neurotransmission and vesicular trafficking (Farsad et al., 2001; Henne et al., 2007; Itoh and De Camilli, 2006; Peter et al., 2004; Shimada et al., 2007). They play an integrative role, together with dynamin and amphiphysin, in vesicle fission in adaptor protein (AP)-2 clathrin-coated vesicles on the plasma membrane (Conner and Schmid, 2003; Ross et al., 2011).

EA2 is a cellular protein that has a C-terminal SH3 domain through which it identifies and interacts with proline-rich domains present on other cellular proteins. It's subcellular localization has been shown to be both cell- and cell cycle phase-dependent (Balshem et al., 2011). It was reported to be localized predominantly in the nuclei of hemopoietic, fibroblast, and epithelial cell lines, but was found to be localized in the cytoplasm in other cells, such as neurons and osteoclasts (Ochoa et al., 2000; Ringstad et al., 1999) . Furthermore, EA2 shows dynamic localization and differential expression levels in various phases of the cell cycle, with the highest levels being observed in G2/M phase, indicating a potential regulatory role in the cell cycle (Balshem et al., 2011). EA2 also reportedly binds to proteins located at leukocyte podosomes, and hence plays a role in leucocyte motility and phagocytosis (Sanchez-Barrena et al., 2012). In addition to its well-established physiological role in endocytosis, recent works have been studying the EA2 involvement in diseases, such as cardiovascular diseases and some cancers (Liu et al., 2016).

In the current study, our aim was to gain a more detailed description of the distribution of EA2 and to investigate its potential involvement in obesity. Results of these studies will increase our understanding of the potential metabolic role(s) of EA2 in diseases related to adipose tissue and disrupted energy homeostasis.

MATERIALS AND METHODS

All experiments were performed at the Obesity Research Center, College of Medicine, King Saud University (KSU), Riyadh, Saudi Arabia, and the Institute of Molecular Biology and Genetics, Department of Biophysics and Chemical Biology, School of Biological Sciences, Seoul National University, Seoul, Korea. The Institutional Review Board in both institutes approved these studies and all participants gave informed consent.

Human EA2 gene expression in paired subcutaneous and visceral adipose tissue

The study population included 24 human adults undergoing elective abdominal surgery for cholecystectomy or weight reduction bariatric surgery. Subjects were free of acute inflammation, infection, or malignancy. All subjects maintained a stable weight, with no fluctuations greater than 2% for at least 2 months prior to surgery, and all subjects underwent a physical examination prior to surgery. Weight in kilograms was measured to the nearest 0.1 kg using a calibrated balance scale, with the subject in light clothing and without shoes. Height was measured using a standing stadiometer to the nearest centimeter. Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meter. Paired subcutaneous and visceral fat samples were obtained from the same donor during the surgical procedure. Tissues were immediately frozen in liquid nitrogen and stored at -80℃ until RNA was extracted.

RNA was extracted from adipose tissue samples using an RNeasy Lipid Tissue kit (Qiagen, USA). Extraction was followed by a DNase digestion step. RNA was quantitated using a Nanodrop ND-1000 spectrophotometer (LabTec, UK). RNA quality was further evaluated via agarose gel electrophoresis and visual inspection of the 28S and 18S ribosomal RNA bands. RNA was reverse-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The synthesized cDNA was used for quantitative real-time PCR (qRT-PCR) and the relative level of the EA2 mRNA expression was detected using a human EA2 primer set and a primer set for the reference gene, Ribosomal Protein L13a (RPL13a) (Curtis et al., 2010; Tsihlias et al., 2000). Primer sequences are available on request. Each reaction contained cDNA, appropriate primers, PCR master mix (containing dNTPs, polymerase, buffer, Cyber Green working solution and glycerol) and water. A negative control to detect DNA contamination was per-

formed by omitting the cDNA. All reactions were carried out in triplicate. Samples were incubated at 94℃ for 3 min for initial denaturation, followed by 40 cycles, each consisting of 94℃ for 30 s, 61.5℃ for 30 s and 72℃ for 20 s, and finally melting for 2 min followed by 5 s at 65℃. A Bio-Rad CFX96 real time system, c1000 thermal cycler, and CFX Manager 2.0 software were used (Bio-Rad, USA). Data were obtained as Ct values (cycle number at which logarithmic PCR plot crosses a calculated threshold line) according to the manufacturer's guidelines. Amplification products were confirmed on a 3% agarose gel stained with EZ-Vision ethidium bromide-free stain (Amresco, USA). Results were correlated to expression levels of other mediators (adiponectin, chemerin, orosomucoid (ORM), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) that were previously assessed using the same cDNA samples (Alfadda et al., 2012a; 2012b).

Human EA2 protein expression in subcutaneous abdominal adipose tissue

Subcutaneous abdominal adipose tissue samples were obtained via liposuction from lean (n = 4) and obese (n = 4), otherwise healthy, non-diabetic adult subjects undergoing elective liposuction surgery. Samples were processed on the day of harvest according to previously published methods (McTernan et al., 2002). Briefly, abdominal fat was digested with collagenase class 1 (2 mg/ml, in 1x HBSS, for 30 min at 37℃) in a shaking water bath. The disrupted tissue was filtered through a sterile double-layered cotton mesh and mature adipocytes were separated from the stromovascular fraction (SVF) via centrifugation (360 x *g*, 5 min, at room temperature). The floating layer of mature adipocytes was removed from the collagenase-dispersed preparation and from the SVF pellet, washed three times in Dulbecco's modified eagle's medium (DMEM, from Hyclone, USA), and stored at -80℃ until analysis. SVF pellets were washed three times in RBCs lysis buffer, resuspended in DMSO freezing-down solution, and stored in liquid nitrogen until analysis.

Cells (mature adipocytes and SVF) were lysed in RIPA buffer and total protein concentration was determined using a BCA protein assay kit (Pierce, USA) according to the manufacturer's instructions. Lysates of mature adipocytes and SVF were separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (PVDF; Millipore Corp., USA). Blots were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline-Tween 20 (TBST) for 1 h. Membranes were incubated overnight with EA2 (mouse monoclonal, SH3GL1; *OriGene* Technologies, Rockville, MD, USA) or β-actin (Cell Signaling Technology Inc., USA) primary antibodies (1:1000 dilution of each antibody) in 1% BSA in TBST. Membranes were washed with TBST prior to addition of a horseradish peroxidase-conjugated secondary antibody (1:5,000; Cell Signaling Technology Inc., USA) for 1 h at room temperature. Membranes were washed with TBST, and a chemiluminescent substrate (Super signal west femto maximum sensitivity substrate kit; Thermo Scientific, USA) was used to visualize antibody binding. Images were captured using GeneSys *image capture* software on a *G:BOX (Syngene, UK)*. Quantitation of protein band intensity, normalized to the intensity of β-actin for each sample, was performed using GeneTools software (Syngene, *UK*). Data are reported as normalized protein band intensity in arbitrary units (AU).

3T3-L1 cell culture and differentiation and siRNA transfection

3T3-L1 cell culture and differentiation were performed as previously described (Lee et al., 2008). All the reagents were from Hyclone (USA). Briefly, 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% bovine calf serum. Two days after confluence, which is considered differentiation day zero (D0), cells were incubated with DMEM supplemented with 10% fetal bovine serum, 3-isobutyl-1-methylxanthine (IBMX; 500 μM), dexamethasone (1 μM), and insulin (5 μg/ml). Two days later, the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and insulin (1 μg/ml) (D2). After this, the culture medium was replaced every other day with DMEM supplemented with 10% fetal bovine serum (D4, and so on). Differentiation was assessed using Oil Red O staining as previously described (Kim et al., 2010). During the differentiation process, cells were harvested on days 0, 2, 4, and 7. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Quantitative RT-PCR was performed as described above. Relative amounts of mRNA were calculated by normalization to β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using the comparative threshold cycle method.

Small interfering RNA duplexes for EA2 were designed and purchased from Bioneer Inc. (Korea). Small interfering RNA (20 μM), were delivered into 3T3-L1 cells using Microporator (Digital Bio Technology Co. Ltd., Korea).

Animal experiments

All experiments were approved by the Seoul National University Animal Experiment Ethics Committee. Male C57BL/6J mice were housed in cages under a 12-h light/12-h dark cycle and controlled temperature (26℃). Two sets of experiments were performed. In the first set, mice were fed either a standard normal chow diet or a high-fat diet (HFD: 60% fat). Mice were sacrificed at the same time, and adipose tissue, both inguinal and epididymal, were dissected. These samples were used to determine the effect of diet on EA2 gene expression in adipose tissue. In the second set of experiments, mice were fed a standard normal chow diet and sacrificed when they reached 18 weeks of age. Several tissues and organs were harvested: adipose tissue [inguinal, epididymal and brown (subscapular)], liver, muscle, kidney, intestine, spleen, heart, brain, pancreas, and lung). Organs were immediately frozen in liquid nitrogen then stored at -80℃ until RNA extraction. These samples were used to determine the tissue-specific gene expression of EA2. RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above.

Statistical analysis

Data are presented as mean ± SEM, unless stated otherwise. The student's *t*-test was used for comparative analysis. Correlation was determined using the Pearson correlation coefficient. P value of ≤ 0.05 was considered statistically significant.

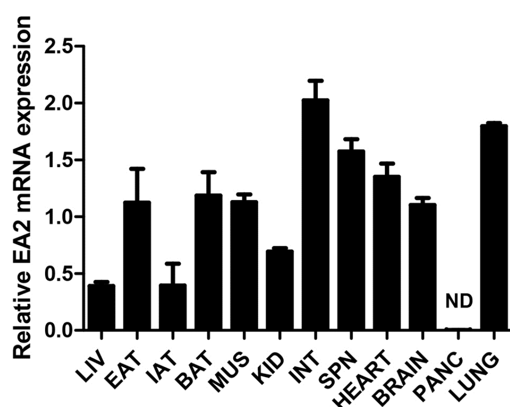

Fig. 1. Relative EA2 gene expression in various tissues of C57BL/6J mice. EA2 gene expression was determined via qRT-PCR and normalized to GAPDH expression. Data represent normalized values. LIV, liver; EAT, epididymal adipose tissue; IAT, inguinal adipose tissue; BAT, brown adipose tissue (subscapular); MUS, muscle; KID, kidney; INT, intestine; SPN, spleen; PANC, pancreas; ND, not detected.

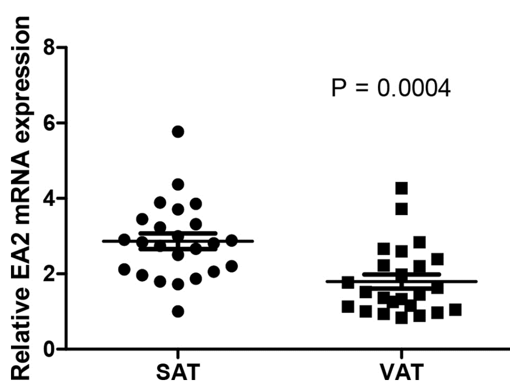

Fig. 2. Relative EA2 gene expression in human adipose tissue. EA2 gene expression in human adipose tissue, in subcutaneous and visceral fat depots (SAT and VAT) was determined via qRT-PCR and normalized to ribosomal protein L13a (RPL13a). Data represent normalized values. P = 0.0004.

IBM SPSS Statistics software for Windows, Version 22.0, IBM Corp. was used for all analyses (IBM Corp., USA).

RESULTS

EA2 gene expressions in various animal tissues and in human paired adipose tissue

EA2 was found to be differentially expressed in various animal tissues, as shown in Fig. 1. Gene expression levels were higher in the intestine, lung, spleen, and heart compared to those in the inguinal adipose tissue, liver, muscle and brain. Low levels of EA2 expression were also observed in the kidney, and epididymal and brown adipose tissue, while no detectable expression was observed in the pancreas.

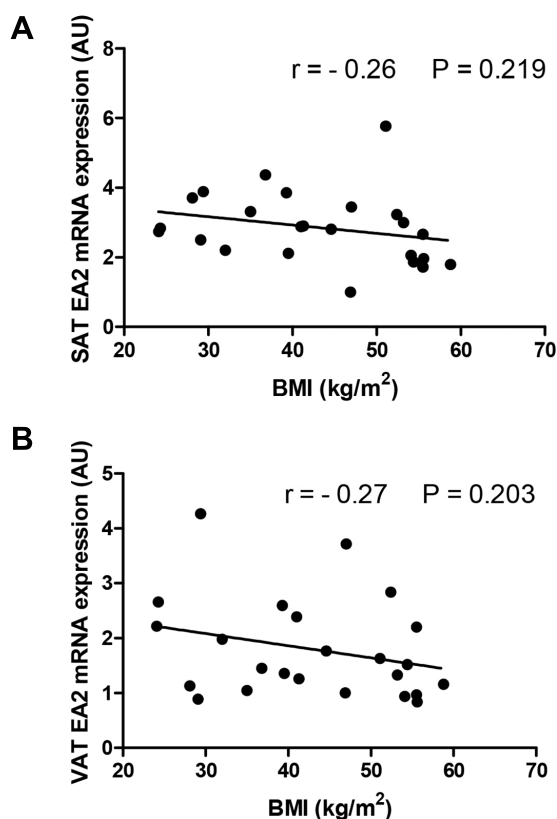

Fig. 3. Correlation between EA2 gene expression in human adipose tissue and body mass index (BMI). (A) SAT (r = -0.26, P = 0.219), and (B) VAT (r = -0.27, P = 0.203).

EA2 gene expression was detected in both human subcutaneous and visceral adipose tissue (SAT and VAT, respectively). Expression levels were significantly higher in SAT than in VAT (P = 0.0004; Fig. 2). Analysis of EA2 gene expression in SAT and VAT in relation to the subjects' calculated BMI values, revealed a negative trend, but did not reach statistical significance (Fig. 3).

EA2 gene expression was then measured in paired adipose tissues and compared to that of other adipokines and inflammatory markers in the same tissues (Alfadda et al., 2012a; 2012b). This revealed a negative correlation between EA2 and two adipokines, adiponectin (Figs. 4A and 4B) and chemerin (Figs. 4C and 4D) in VAT but not in SAT. A positive correlation was detected between EA2 and TNF-α expression in SAT but not in VAT (Figs. 4E and 4F). No significant correlation was observed between gene expression levels of EA2 and ORM or IL-6 in both SAT and VAT (data not shown).

EA2 protein expression in human mature adipocytes

To further understand the distribution of EA2, we evaluated its protein expression in adipose tissue from lean and obese subjects. Mature adipocytes were separated from human subcutaneous abdominal adipose tissue obtained via liposuction from four lean individuals (average BMI ± SD = 24.2

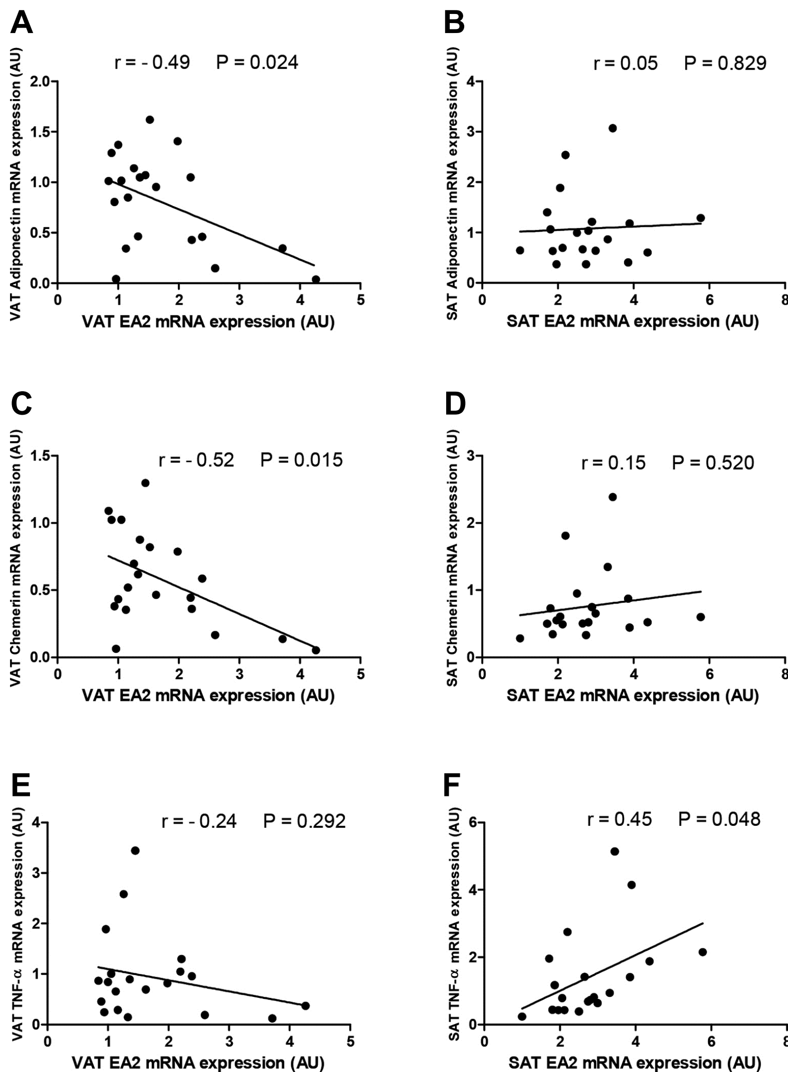

Fig. 4. Correlation between EA2 gene expression in SAT and VAT and adiponectin (A & B), chemerin (C & D), and TNF-α (E & F).

kg/m^2 ± 0.71) and four obese individuals (average BMI ± SD = 42.6 kg/m^2 ± 3.4) and EA2 protein expression levels were measured using immunoblotting (Fig. 5A). Densitometric analysis of the immunoblotting data revealed higher EA2 protein expression in mature adipocytes isolated from obese compared to that in lean individuals. However, this difference did not reach statistical significance (Fig. 5B). On the contrary, no difference was detected in EA2 protein expression levels in SVF from lean and obese individuals (data not shown).

EA2 gene expression during 3T3-L1 preadipocyte differentiation

To determine whether EA2 expression changes during adipocyte differentiation, the 3T3-L1 cell line was used. As shown in Fig. 6, EA2 was expressed at all stages of differentiation. However, a dramatic decrease in expression levels was observed as differentiation progressed, with the lowest levels seen on day 7 of differentiation (P = 0.02).

EA2 suppression in adipocytes and its effect on inflammatory markers

To clarify the role of EA2 in adipocyte inflammatory response, we suppressed adipocyte EA2 by using siRNA. There was a significant decrease in the gene expression of IL-6 (P = 0.02), and in Monocyte Chemoattractant Protein-1(MCP-1) (P = 0.002) in EA2-suppressed adipocytes (Fig. 7).

EA2 gene expression in fat depots obtained from C57BL/6 mice fed a normal chow diet or a high fat diet

To determine the levels of EA2 gene expression in the experimental animal fat depot, and whether this is influenced by the dietary regimen, we used two groups of C57BL/6 mice; one fed a normal chow diet and the other fed a high fat diet. EA2 gene expression levels were measured in inguinal and epididymal fat depots in both groups. Our results demonstrate that the type of diet had no influence on EA2 expression levels in both fat depots (data not shown).

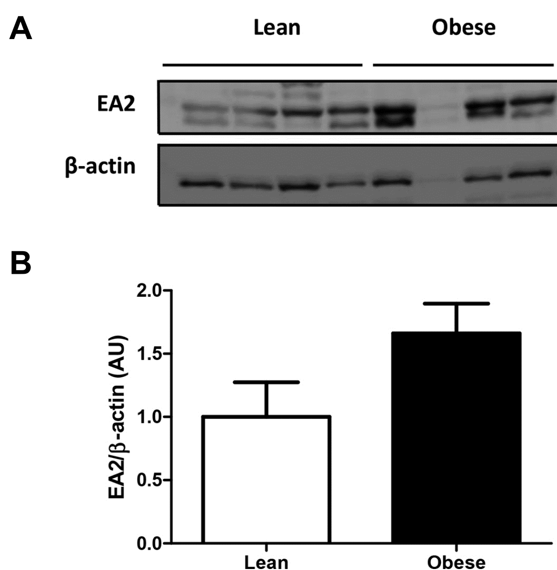

Fig. 5. EA2 protein expression in human mature adipocytes isolated from abdominal liposuction adipose tissue of lean (n = 4) and obese (n = 4) subjects. (A) Immunoblotting of proteins extracted from human mature adipocytes using anti-EA2 and anti-β-actin antibodies. (B) Graphical representation of relative intensity values of normalized EA2 protein bands for lean and obese individuals. Data are represented as a bar graph of mean ± SEM.

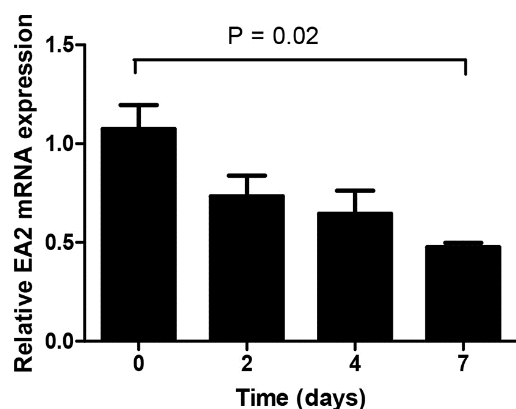

Fig. 6. EA2 gene expression in 3T3-L1 cells at different stages of adipocyte differentiation. EA2 gene expression measured via qRT-PCR from the adipocyte cell line 3T3-L1 in the undifferentiated state (day 0), and during differentiation (days 2, 4, and 7). P = 0.02. Data are represented as a bar graph of mean ± SEM.

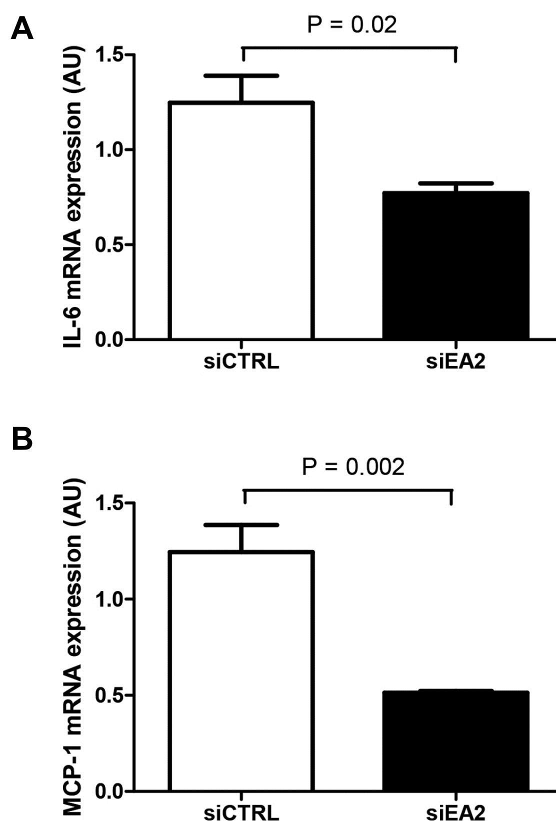

Fig. 7. IL-6 and MCP-1 gene expression in adipocyte after suppression of EA2 with siRNA as compared to a control siRNA (siCTRL). (A) IL-6 gene expression (P = 0.02), and (B) MCP-1 gene expression (P = 0.002) were measured in 3T3-L1 cells via qRT-PCR. Data are represented as a bar graph of mean ± SEM.

DISCUSSION

The present study revealed the distribution of EA2 in fat depots and its correlation with other mediators in adipose tissue. The data suggest a potential role for EA2 in obesity and associated co-morbidities. We evaluated EA2 gene expression in human SAT and VAT, in a 3T3-L1 cell line during adipocyte differentiation, and in tissues isolated from experimental animals. In addition, EA2 gene expression was analyzed relative to the expression of other adipokines and inflammatory mediators, and the effects of EA2 silencing on the expression of well-established inflammatory mediators was evaluated. EA2 expression in human adipose tissue was also confirmed at the protein level. To the best of our knowledge, there have been no reports thus far on the expression of EA2 in human adipose tissue, at either the gene or the protein level.

Our finding that EA2 is differentially expressed in fat depots, both in humans and in rodents, could be an indication of its key role in these tissues. EA2 protein represents one component of an adaptor protein complex that forms an interposing signal between cell surface receptor kinases and more downstream signaling cascade molecules. EA2 is also capable of locating small GTPases to its site of action on the membrane (Bouwman et al., 2014). It is therefore possible that the structure of EA2 allows it to play a critical role in the differences between SAT and VAT, and that it could have novel functions in adipose tissue, beyond its well established role in mediating endocytosis. Consistent with our current findings, other mediators have been shown to be differentially expressed in SAT vs. VAT, implying that either these

mediators play different roles or that an entirely different regulatory mechanism is in place in these tissues (Alfadda et al., 2012b; Alessi et al., 1997; Fried et al., 1998; Montague et al., 1998; Samaras et al., 2010).

The differences in EA2 gene expression levels in the fat depot relative to that of other adipokines, such as adiponectin and chemerin, are interesting. Adiponectin is a known anti-inflammatory adipokine, while chemerin is thought to exert dual roles, depending on the cleavage product and hence the predominant molecular form (Alfadda et al., 2012b). A negative correlation between EA2 and the anti-inflammatory adipokine, adiponectin, and a positive correlation between EA2 and the inflammatory marker, TNF-α, suggests it may play a pro-inflammatory role. Further support of this role in the current work is demonstrated by 2 findings, first, the significant suppression of the gene expression of two of the well-documented inflammatory mediators, IL-6 and MCP-1, upon suppression of EA2 in mature 3T3-L1 cells, and second, the increased protein expression levels in SAT obtained from obese vs. lean individuals. Interestingly, a recent work by Huang and co-workers reported a novel role for EA2 in macrophage-derived foam cell formation, which is an inflammation-driven process critical to the pathogenesis of atherosclerosis. The investigators demonstrated that oxidized LDL (oxLDL) triggered interaction of EA2 with the macrophages' scavenger receptors, resulting in activation of inflammation-related signaling pathways that increased expression of the scavenger receptors leading to more oxLDL uptake and hence, foam cell formation. Furthermore, and similar to our finding of the anti-inflammatory consequence of silencing EA2, foam cell formation was downregulated by EA2 inhibition (Huang et al., 2016). These findings support a role for EA2 in inflammation-related diseases; and are in line with recent works linking EA2 to cardiovascular diseases (Liu et al., 2016), and to certain aggressive forms of breast cancer with poor prognosis (Baldassarre et al., 2015).

The observed negative trend between BMI and EA2 gene expression in human paired adipose tissue does, however, seem inconsistent with the increasing trend in EA2 protein levels in adipose tissue samples from obese relative to lean individuals. Although interpreting these findings is beyond the objectives of the current study, which was designed to provide a descriptive analysis of EA2 expression in adipose tissue, it does suggest the presence of a feedback mechanism, a hypothesis that should be tested in future studies. It is possible that high EA2 protein levels lead to a downregulation of gene expression level in adipose tissues, in a negative feedback manner. Alternatively, and based on the known imperfect relationship between corresponding mRNA and protein abundances, it might be relevant to note that mRNA level is capable to explain only a fraction of the protein abundance in various species and cells, while ~ 1/3 - 2/3 of the variations in protein's abundance are explained by other factors; including post-transcriptional, translational, post-translational, and protein stability factors (Vogel and Marcotte, 2012). Therefore, a negative trend between the EA2 gene expression and BMI needs to be studied in further details in the future; taking into account various potential contributing factors.

Our finding the EA2 gene expression is downregulated during 3T3-L1 preadipocyte differentiation is interesting. However, it may not be easily extrapolated to human adipose tissue due to its cellular and functional complexity. Nevertheless, the published role for EA2 in regulating cell division, specifically at the G2/M phase of the cell cycle (Balshem et al., 2011), is a possible link that would require further analysis. In addition, a recent proteomics study demonstrated significant downregulation of another member of the endophilin family, endophilin-B1, during the differentiation of mouse primary brown preadipocytes (Kamal et al., 2013). Thus, studying EA2 gene and protein expression levels during human preadipocyte differentiation will be required to clarify the role of EA2 in human adipogenesis.

One possible link to the suggested role of EA2 in adipogenesis could be its well-known role in signaling pathways and in integrative crosstalk between endosomes. We propose that EA2 would affect multiple metabolic pathways, based on its critical location and interaction with components of signaling pathways that would allow it to participate in crosstalk in many interactive biological systems. In support of this are earlier studies regarding the discovery and characterization of EA2 and other members of the endophilin family (Soubeyran et al., 2002; Tang et al., 1999; Yam et al., 2004). For example, a novel protein, termed EA2-binding partner, has been shown to bind EA2 and cause inhibitory effects on the Ras signaling pathway, which is known to regulate cellular transformation (Yam et al., 2004). In addition, more recently published data demonstrate that EA2 binds to proteins known to mediate endosomal crosstalk, such as AXIN (Palfy et al., 2012).

EA2 has remarkable structural and functional characteristics. Structurally, EA2 belongs to a larger family of proteins that possess SH3 domains, the ligands for which have not been fully characterized. The SH3 domain provides these proteins with an influential position in terms of complex formation based on their capability to recognize and bind to a large number of ligands. Saksela and Permi recently published a review article summarizing the nature of interactions between SH3 domain-containing proteins and their ligands, through what they term "specificity zone surfaces" of SH3 domains. They demonstrated that a ligand for the SH3 domain is no more restricted to the consensus peptide, which contains a characteristic proline-rich motif and a basic residue, but rather that a more complex binding interface is required. This in turn provides a more extensive contact surface, and hence a substantially greater binding affinity and selectivity (Saksela and Permi, 2012). Functionally, in addition to their well-known endocytic roles, members of the endophilin family have been linked to several non-endocytic functions. As mentioned above, their role in signaling pathways and receptor interactions and expression is of particular interest (Huang et al., 2016; Tang et al., 1999).

In conclusion, the present study describes the distribution of human EA2 in adipose tissue, and highlights several important, and previously unidentified, correlations between this protein and certain adipokines and mediators of chronic inflammation. The EA2 gene was found to be differentially expressed in human adipose tissue, with more expression in

SAT than VAT, and showed a negative correlation with BMI. In addition, EA2 gene expression negatively correlated with the expression of adiponectin and chemerin in VAT, whereas in SAT, it showed a positive correlation with TNF-α expression. Moreover, silencing EA2 in 3T3-L1 cells significantly downregulated the gene expression of IL-6 and MCP-1. Furthermore, EA2 protein expression was higher in mature adipocytes obtained from obese subjects compared to that in lean subjects. Interestingly, the EA2 gene expression was found to be downregulated during adipocyte differentiation in a murine cell line. Collectively, the current study provides a more comprehensive description of EA2 distribution, reveals new correlations with other molecules, and emphasizes the need to study this protein further in the context of adipogenesis and adipocytes signaling pathways and metabolism.

ACKNOWLEDGMENTS

The authors acknowledge the technical help of Mrs. Ruba Elawad, Mr. Kenneth Domero, and Mr. Shahid Nawaz. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this research through the Research Group Project no. RGP-334.

REFERENCES

Alessi, M.C., Peiretti, F., Morange, P., Henry, M., Nalbone, G., and Juhan-Vague, I. (1997). Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link between visceral fat accumulation and vascular disease. Diabetes *46*, 860-867.

Alfadda, A.A., Fatma, S., Chishti, M.A., Al-Naami, M.Y., Elawad, R., Mendoza, C.D., Jo, H., and Lee, Y.S. (2012a). Orosomucoid serum concentrations and fat depot-specific mRNA and protein expression in humans. Mol. Cells *33*, 35-41.

Alfadda, A.A., Sallam, R.M., Chishti, M.A., Moustafa, A.S., Fatma, S., Alomaim, W.S., Al-Naami, M.Y., Bassas, A.F., Chrousos, G.P., and Jo, H. (2012b). Differential patterns of serum concentration and adipose tissue expression of chemerin in obesity: adipose depot specificity and gender dimorphism. Mol. Cells *33*, 591-596.

Alfadda, A.A., Turjoman, A.A., Moustafa, A.S., Al-Naami, M.Y., Chishti, M.A., Sallam, R.M., Gibson, D., and Duncan, M.W. (2014). A proteomic analysis of excreted and circulating proteins from obese patients following two different weight-loss strategies. Exp. Biol. Med. (Maywood) *239*, 568-580.

Baldassarre, T., Watt, K., Truesdell, P., Meens, J., Schneider, M.M., Sengupta, S.K., and Craig, A.W. (2015). Endophilin A2 promotes TNBC cell invasion and tumor metastasis. Mol. Cancer Res. *13*, 1044-1055.

Balshem, H., Helfand, M., Schunemann, H.J., Oxman, A.D., Kunz, R., Brozek, J., Vist, G.E., Falck-Ytter, Y., Meerpohl, J., Norris, S., et al. (2011). GRADE guidelines: 3. Rating the quality of evidence. J. Clin. Epidemiol. *64*, 401-406.

Bouwman, F.G., Claessens, M., van Baak, M.A., Noben, J.P., Wang, P., Saris, W.H., and Mariman, E.C. (2009). The physiologic effects of caloric restriction are reflected in the *in vivo* adipocyte-enriched proteome of overweight/obese subjects. J. Proteome Res. *8*, 5532-5540.

Bouwman, F.G., Wang, P., van Baak, M., Saris, W.H., and Mariman, E.C. (2014). Increased beta-oxidation with improved glucose uptake capacity in adipose tissue from obese after weight loss and maintenance. Obesity *22*, 819-827.

Cao, H. (2014). Adipocytokines in obesity and metabolic disease. J. Endocrinol. *220*, T47-59.

Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. Nature *422*, 37-44.

Curtis, K.M., Gomez, L.A., Rios, C., Garbayo, E., Raval, A.P., Perez-Pinzon, M.A., and Schiller, P.C. (2010). EF1alpha and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells. BMC Mol. Biol. *11*, 61.

Farsad, K., Ringstad, N., Takei, K., Floyd, S.R., Rose, K., and De Camilli, P. (2001). Generation of high curvature membranes mediated by direct endophilin bilayer interactions. J. Cell Biol. *155*, 193-200.

Fried, S.K., Bunkin, D.A., and Greenberg, A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J. Clin. Endocrinol. Metabol. *83*, 847-850.

Gu, Y., Zhao, A., Huang, F., Zhang, Y., Liu, J., Wang, C., Jia, W., and Xie, G. (2013). Very low carbohydrate diet significantly alters the serum metabolic profiles in obese subjects. J. Proteome Res. *12*, 5801-5811.

Henne, W.M., Kent, H.M., Ford, M.G., Hegde, B.G., Daumke, O., Butler, P.J., Mittal, R., Langen, R., Evans, P.R., and McMahon, H.T. (2007). Structure and analysis of FCHo2 F-BAR domain: a dimerizing and membrane recruitment module that effects membrane curvature. Structure *15*, 839-852.

Hotamisligil, G.S. (2006). Inflammation and metabolic disorders. Nature *444*, 860-867.

Huang, E.W., Liu, C.Z., Liang, S.J., Zhang, Z., Lv, X.F., Liu, J., Zhou, J.G., Tang, Y.B., and Guan, Y.Y. (2016). Endophilin-A2-mediated increase in scavenger receptor expression contributes to macrophage-derived foam cell formation. Atherosclerosis *254*, 133-141.

Itoh, T., and De Camilli, P. (2006). BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim. Biophys. Acta *1761*, 897-912.

Kamal, A.H., Kim, W.K., Cho, K., Park, A., Min, J.K., Han, B.S., Park, S.G., Lee, S.C., and Bae, K.H. (2013). Investigation of adipocyte proteome during the differentiation of brown preadipocytes. J. Proteomics *94*, 327-336.

Kim, S.Y., Kim, A.Y., Lee, H.W., Son, Y.H., Lee, G.Y., Lee, J.W., Lee, Y.S., and Kim, J.B. (2010). miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochem. Biophys. Res. Commun. *392*, 323-328.

Kjaerulff, O., Brodin, L., and Jung, A. (2011). The structure and function of endophilin proteins. Cell Biochem. Biophys. *60*, 137-154.

Lee, Y.S., Kim, A.Y., Choi, J.W., Kim, M., Yasue, S., Son, H.J., Masuzaki, H., Park, K.S., and Kim, J.B. (2008). Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress. Mol. Endocrinol. *22*, 2176-2189.

Liu, C.Z., Li, X.Y., Du, R.H., Gao, M., Ma, M.M., Li, F.Y., Huang, E.W., Sun, H.S., Wang, G.L., and Guan, Y.Y. (2016). Endophilin A2 influences volume-regulated chloride current by mediating ClC-3 trafficking in vascular smooth muscle cells. Circ. J. *80*, 2397-2406.

McGown, C., Birerdinc, A., and Younossi, Z.M. (2014). Adipose tissue as an endocrine organ. Clin. Liver Dis. *18*, 41-58.

McTernan, P.G., Anderson, L.A., Anwar, A.J., Eggo, M.C., Crocker, J., Barnett, A.H., Stewart, P.M., and Kumar, S. (2002). Glucocorticoid regulation of p450 aromatase activity in human adipose tissue: gender and site differences. J. Clin. Endocrinol. Metabol. *87*, 1327-1336.

Montague, C.T., Prins, J.B., Sanders, L., Zhang, J., Sewter, C.P., Digby,

J., Byrne, C.D., and O'Rahilly, S. (1998). Depot-related gene expression in human subcutaneous and omental adipocytes. Diabetes *47*, 1384-1391.

O'Rahilly, S. (2016). Harveian Oration 2016: Some observations on the causes and consequences of obesity. Clin. Med. *16*, 551-564.

Ochoa, G.C., Slepnev, V.I., Neff, L., Ringstad, N., Takei, K., Daniell, L., Kim, W., Cao, H., McNiven, M., Baron, R., et al. (2000). A functional link between dynamin and the actin cytoskeleton at podosomes. J. Cell Biol. *150*, 377-389.

Palfy, M., Remenyi, A., and Korcsmaros, T. (2012). Endosomal crosstalk: meeting points for signaling pathways. Trends Cell Biol. *22*, 447-456.

Peter, B.J., Kent, H.M., Mills, I.G., Vallis, Y., Butler, P.J., Evans, P.R., and McMahon, H.T. (2004). BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science *303*, 495-499.

Ringstad, N., Gad, H., Low, P., Di Paolo, G., Brodin, L., Shupliakov, O., and De Camilli, P. (1999). Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. Neuron *24*, 143-154.

Romacho, T., Elsen, M., Rohrborn, D., and Eckel, J. (2014). Adipose tissue and its role in organ crosstalk. Acta Physiol (Oxf) *210*, 733-753.

Ross, J.A., Chen, Y., Muller, J., Barylko, B., Wang, L., Banks, H.B., Albanesi, J.P., and Jameson, D.M. (2011). Dimeric endophilin A2 stimulates assembly and GTPase activity of dynamin 2. Biophys. J. *100*, 729-737.

Saksela, K., and Permi, P. (2012). SH3 domain ligand binding: What's the consensus and where's the specificity? FEBS Lett. *586*, 2609-2614.

Samaras, K., Botelho, N.K., Chisholm, D.J., and Lord, R.V. (2010). Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes. Obesity (Silver Spring) *18*, 884-889.

Sanchez-Barrena, M.J., Vallis, Y., Clatworthy, M.R., Doherty, G.J., Veprintsev, D.B., Evans, P.R., and McMahon, H.T. (2012). Bin2 is a membrane sculpting N-BAR protein that influences leucocyte podosomes, motility and phagocytosis. PLoS One *7*, e52401.

Shimada, A., Niwa, H., Tsujita, K., Suetsugu, S., Nitta, K., Hanawa-Suetsugu, K., Akasaka, R., Nishino, Y., Toyama, M., Chen, L., et al. (2007). Curved EFC/F-BAR-domain dimers are joined end to end into a filament for membrane invagination in endocytosis. Cell *129*, 761-772.

Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W.Y., and Dikic, I. (2002). Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. Nature *416*, 183-187.

Speakman, J.R., and Mitchell, S.E. (2011). Caloric restriction. Mol. Aspects Med. *32*, 159-221.

Suganami, T., Nishida, J., and Ogawa, Y. (2005). A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Arterioscler. Thromb. Vasc. Biol. *25*, 2062-2068.

Sun, K., Kusminski, C.M., and Scherer, P.E. (2011). Adipose tissue remodeling and obesity. J. Clin. Invest. *121*, 2094-2101.

Tang, Y., Hu, L.A., Miller, W.E., Ringstad, N., Hall, R.A., Pitcher, J.A., DeCamilli, P., and Lefkowitz, R.J. (1999). Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta1-adrenergic receptor. Proc. Natl. Acad. Sci. USA *96*, 12559-12564.

Tsihlias, E.B., Gibbs, A.L., McBurney, M.I., and Wolever, T.M. (2000). Comparison of high- and low-glycemic-index breakfast cereals with monounsaturated fat in the long-term dietary management of type 2 diabetes. Am. J. Clin. Nutr. *72*, 439-449.

Vogel, C., and Marcotte, E.M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. *13*, 227-232.

Wellen, K.E., and Hotamisligil, G.S. (2005). Inflammation, stress, and diabetes. J. Clin. Invest. *115*, 1111-1119.

Yam, J.W., Jin, D.Y., So, C.W., and Chan, L.C. (2004). Identification and characterization of EBP, a novel EEN binding protein that inhibits Ras signaling and is recruited into the nucleus by the MLL-EEN fusion protein. Blood *103*, 1445-1453.