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Identification of anti-adipogenic proteins in adult bovine serum suppressing 3T3-L1 preadipocyte differentiation

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Adipocyte differentiation is a complex developmental process forming adipocytes from various precursor cells. The murine 3T3-L1 preadipocyte cell line has been most frequently used in the studies of adipocyte differentiation. Differentiation of 3T3-L1 preadipocytes includes a medium containing fetal bovine serum (FBS) with hormonal induction. In this study, we observed that differentiation medium containing adult bovine serum (ABS) instead of FBS did not support differentiation of preadipocytes. Impaired adipocyte differentiation was due to the presence of a serum protein factor in ABS that suppresses differentiation of preadipocytes. Using a proteomic analysis, alpha-2-macroglobulin and paraoxonase/arylesterase 1, which were previously shown to suppress differentiation of preadipocytes, were identified as anti-adipogenic proteins. Although their functional mechanisms have not yet been elucidated, the anti-adipogenic effects of these proteins are discussed. [BMB Reports 2013; 46(12): 582-587]

INTRODUCTION

Adipocytes have the capacity to accumulate lipids contributing to energy homeostasis. However, over proliferation of adipocytes results in development of obesity and obesity-related diseases such as type 2 diabetes and atherosclerosis (1, 2). Adipocytes are formed by differentiation of multipotent mesenchymal stem cells or other adipogenic precursor cells. Therefore adipocyte differentiation has been extensively studied in order to elucidate the underlying molecular mechanism. Many types of primary adipogenic cells and established preadipocyte cell lines have been used for studying adipocyte differentiation. However, considerable progress has been made with the murine 3T3-L1 preadipocyte cell line, which is most frequently used to screen the adipogenic or anti-adipogenic potential of various reagents (3).

The standard protocol for differentiation of 3T3-L1 preadipocyte includes a defined medium containing fetal bovine serum (FBS) with hormonal induction by the addition of an adipogenic cocktail that generally consists of 3-isobutyl-1-methylxanthine, dexamethasone and insulin (3). FBS is a crucial component in the culture medium of most mammalian cells, and provides complex biological molecules such as hormones, growth factors, and numerous low molecular weight nutrients. For the differentiation of 3T3-L1 preadipocyte, it was shown that FBS is far more effective than calf serum and sera of other animal species, suggesting that FBS may contain an adipogenic factor (4). In another reports, horse serum was shown to be more effective than FBS for the differentiation of adipogenic stem cells into adipocytes (5, 6). Recently, we also showed that adult bovine serum (ABS) has the capacity to regulate lipid accumulation in myogenic precursor cells during trans-differentiation into adipocytes (7). In addition, ABS and FBS were characterized to possess different contents of lipids, proteins and hormones, although serum constituents could not be fully defined (7, 8). These results have suggested that ABS may contain a distinct serum factor that regulates the differentiation of adipogenic precursor cells (8).

In the present study, we found that differentiation of 3T3-L1 preadipocyte was significantly diminished, when ABS replaced FBS in the differentiation medium. Suppression of differentiation appeared to be, at least in part, due to the presence of an anti-adipogenic protein(s) in ABS. Two putative anti-adipogenic proteins, alpha-2-macroglobulin and paraoxonase/arylesterase 1, which were previously shown to suppress adipocyte differentiation (9) or involved in lipid metabolism and accumulation (10), were identified by proteomic analysis. Based on these results, the anti-adipogenic functions of the identified proteins are discussed.

RESULTS

Impaired differentiation of preadipocytes in ABS medium

Differentiation of post-confluent 3T3-L1 preadipocytes was induced by hormonal stimulation in medium containing fetal bovine serum (FBS) or adult bovine serum (ABS). Preadipocytes

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differentiated in FBS medium showed typical adipogenic morphological changes with accumulation of intracellular lipid droplets (Fig. 1A). In contrast, hormonal stimulation in ABS medium did not induce either a significant change in cell morphology or accumulation of lipid droplets, indicating impaired differentiation of preadipocytes. Determination of intracellular lipid contents by oil red-O staining clearly showed that differentiation of preadipocytes in ABS medium was dramatically diminished (Fig. 1B), as compared with differentiation in FBS medium. Impaired differentiation of preadipocytes was also evaluated by determining the expression levels of adipogenic marker genes. The mRNA level of an adipogenic transcription factor, PPARγ, was over 2-fold lower in preadipocytes differentiated in ABS medium than those differentiated in FBS medium (Fig. 1E). Consistently, only minor mRNA expression of the PPARγ target genes CD36 and FABP4 was detected in preadipocytes differentiated in ABS medium.

ABS proteins suppress differentiation of preadipocytes

Determination of the serum protein concentration revealed that ABS contained more proteins (~60 mg/ml) than FBS (~30 mg/ml). Analysis of serum proteins by SDS-PAGE consistently showed extra protein bands in ABS that were not detected in FBS (Fig. 1C). Therefore, we hypothesized that an ABS-specific protein(s) may mediate an anti-adipogenic effect that suppresses preadipocyte differentiation in ABS medium. To test this hypothesis, serum proteins were fractionated by partial protein precipitation with addition of polyethylene glycol (PEG, 10% (w/v)). The protein precipitates from ABS and FBS were reconstituted by dissolving in PBS, resulting in the protein solutions ABSppt and FBSppt, respectively. SDS-PAGE analysis showed that the protein content of ABSppt was significantly different from that of FBSppt (Fig. 1C), whereas the protein contents of the supernatants, FBSsup and ABSsup, appeared to be similar.

The effects of fractionated serum proteins on preadipocyte differentiation were tested by supplementing differentiation media with the same amount of ABSppt or FBSppt (Fig. 1D). As determined by oil red-O staining, FBSppt supplementation did not cause any significant change in the differentiation of preadipocytes in FBS or ABS medium. In contrast, ABSppt supplementation caused a significant dose-dependent decrease in preadipocyte differentiation in FBS medium, although the decrease in differentiation level was less than that in ABS medium. To confirm the anti-adipogenic effect of ABSppt, the expression levels of adipogenic marker genes were determined. ABSppt supplementation significantly reduced both the expression of PPARγ as well as the expressions of CD36 and FABP4 in preadipocytes differentiated in FBS medium (Fig. 1E). These results indicate that the impaired differentiation of preadipocytes in ABS medium was, at least in part, due to the presence of an anti-adipogenic ABS protein that was fractionated into ABSppt.

Identification of anti-adipogenic proteins

The proteins in ABSppt and FBSppt were separated by 2D-PAGE and compared with each other in order to screen and identify an anti-adipogenic protein in ABSppt. Eleven pro-

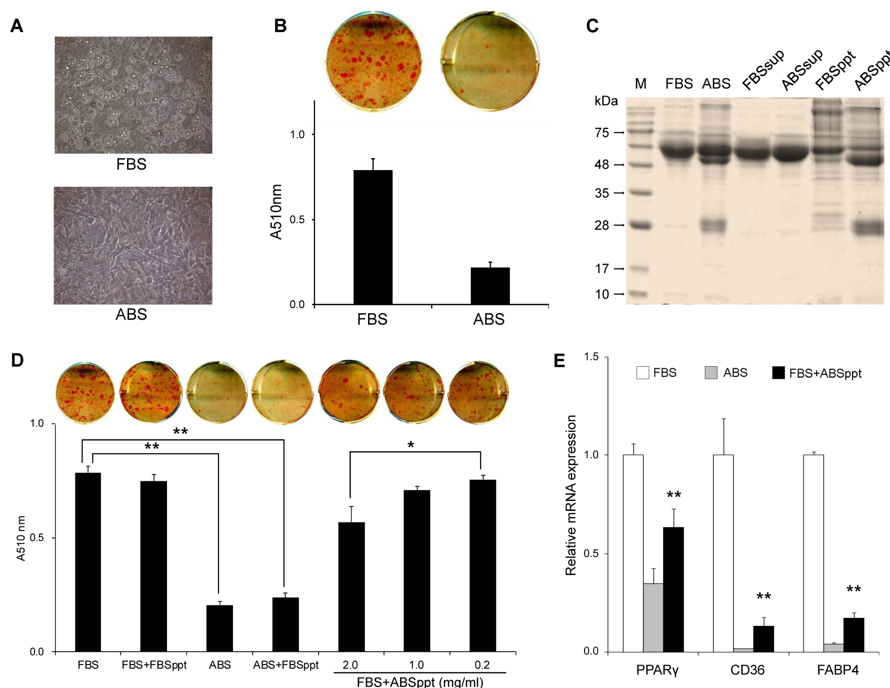

Fig. 1. Suppression of adipocyte differentiation by ABS proteins. 3T3-L1 preadipocytes were differentiated in FBS or ABS medium. After differentiation, cells were observed under a microscope (A) and the lipid contents were determined by oil-red O staining (B). Serum proteins (20 μg each) were analyzed by 15% SDS-PAGE (C): M, molecular size marker; FBS and ABS, corresponding serum proteins; FBSsup and ABSsup, supernatant from PEG fractionation; FBSppt and ABSppt, precipitated proteins from PEG fractionation. Preadipocytes were differentiated in FBS or ABS medium supplemented with FBSppt (2.0 mg/ml medium) or ABSppt at the indicated concentrations in medium, and the lipid contents were determined by oil-red O staining (D). Relative mRNA expression of adipogenic genes in the indicated medium conditions was determined by RT-PCR on day 5 of differentiation induction (E). Data values in D and E are an average of $n \geq 5$ and ex-pressed as means ± SE: *P value <0.01; **P value <0.001.

Table 1. Summary of identified ABSppt-specific proteins by peptide mass finger printing

Spot #	Protein ID	Proteins	Sequence coverage (%)	Score	pI	MW (Da)
1, 2	NP_001103265	Alpha-2-macroglobulin	28	245	5.71	168953
3	NP_001039734	Serum paraoxonase /arylesterase 1	40	108	5.24	40044

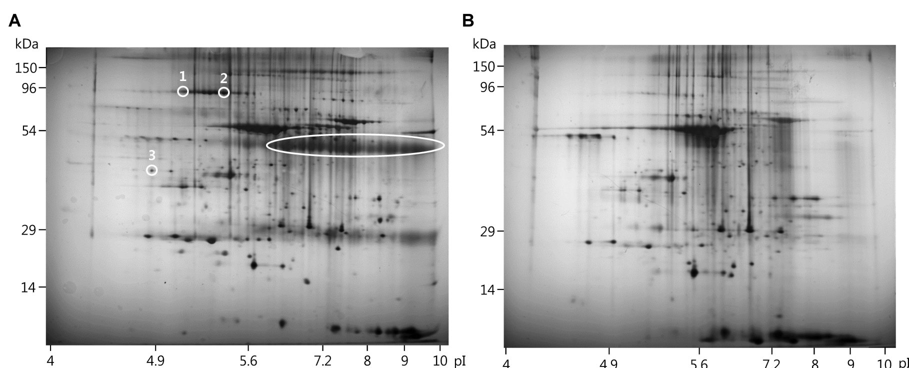

Fig. 2. Identification of anti-adipogenic proteins. Proteins in ABSppt (A) and FBSppt (B) were separated by 2D-PAGE and compared with each other. Proteins detected only in ABSppt were screened and further identified by peptide mass finger printing, as described in the supplementary materials and methods. Identified ABSppt-specific protein are indicated with circles or oval in A and summarized in Table 1.

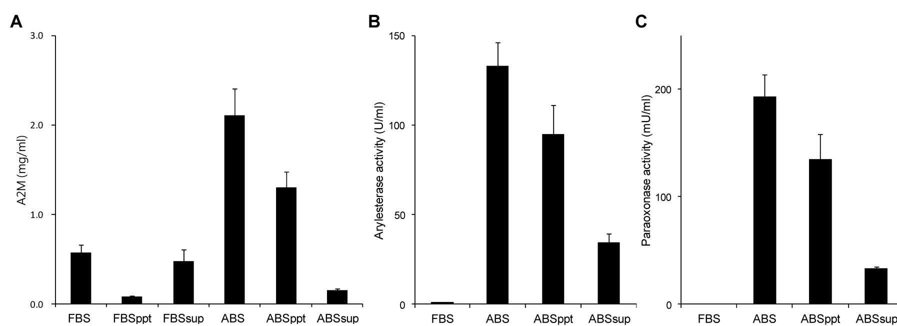

Fig. 3. Determination of A2M and PON1 contents. A2M concentrations (A) were determined based on trypsin binding activity. PON1 concentrations were indirectly determined by measuring arylesterase (B) and paraoxonase activities (C). Sample labels are as described in Fig. 1C. Data for ABSppt and FBSppt were obtained with 1x protein solutions and compared with data for other samples (n $\geq$ 3).

tein spots were detected in ABSppt, but not in FBSppt. Each ABSppt-specific protein was >0.3% of the total ABSppt protein content in terms of protein spot intensity. The ABSppt-specific protein spots were further identified by peptide mass finger printing (Fig. 2 and Table 1). Two protein spots could not be identified due to unknown reasons. Most of the ABSppt-specific protein spots (indicated in Fig. 2 with an oval) were identified as peptides derived from immunoglobulins, which are supposed to be involved in immune responses. However, two protein spots were identified to as alpha-2-macroglobulin (A2M) and paraoxonase/arylesterase 1 (PON1), which were previously reported to suppress adipocyte differentiation (9, 11, 12). Therefore, A2M and PON1 were further analyzed with ABS, FBS, and fractionated serum proteins.

A2M is more abundant in ABS than FBS

The concentration of A2M was determined to be 2.1 mg/ml in ABS, which was 3.5-fold higher than its concentration in FBS (0.6 mg/ml) (Fig. 3A). Further, the concentration of A2M was determined to be 1.3 mg/ml in ABSppt (13.8 μg/mg protein), which was 62% of the total A2M concentration in ABS. However, the A2M concentration in FBSppt was 0.04 mg/ml (2.0 μg/mg protein), which was 7% of the total A2M concentration in FBS.

PON1 is an ABS-specific protein

The concentrations of PON1 in ABS and FBS were estimated by measuring its arylesterase and paraoxonase activities (13). The arylesterase activity of ABS was determined to be 133 U/ml (0.6 U/mg protein), whereas only minor arylesterase activity was detected in FBS (<1 U/ml) (Fig. 3B). The paraoxonase activity of ABS was determined to be 193 mU/ml (0.4 mU/mg protein), whereas no paraoxonase activity was detected in FBS (Fig. 3C). ABSppt exhibited arylesterase activity of 95 U/ml (1.0 U/mg protein) and paraoxonase activity of 135 mU/ml (0.7 U/mg protein), indicating that ∼70% of PON1 in ABS was fractionated into ABSppt.

DISCUSSION

In the present study, we discovered that differentiation of 3T3-L1 preadipocytes was almost completely impaired in medium containing ABS. This observation is consistent with a previous result in which FBS was shown to be far more effective than other animal sera for the differentiation of preadipocytes (4). The authors suggested the presence of an adipogenic factor in FBS that is absent from other animal sera. The adipogenic factor in FBS is unlikely a lipid molecule, as delipidation was shown to not decrease the adipogenic ability of the serum (4). Based on these results, we assumed that FBS may contain a protein(s) with an adipogenic effect or, alternatively, ABS may contain a protein(s) with an anti-adipogenic effect.

The identification of a serum protein is extremely difficult in practice, as serum includes complex proteins. In addition, a few serum proteins such as albumin, immunoglobulins and transferrin are highly abundant constituting $>60\%$ of the total protein content of serum (14). Accordingly, these proteins need to be depleted in order to identify an interesting serum protein of low-abundance. Polyethylene glycol (PEG) is known to neither interact with proteins, nor cause significant changes in protein functions (15). Hence protein precipitation using PEG and reconstitution are frequently used for the fractionation of complex protein mixtures. Fractionation of bovine serum proteins using 10% (w/v) PEG appeared to be efficient for separating highly abundant proteins, which were mostly left in the supernatant (Fig. 1C). The putative adipogenic proteins in FBS were unlikely precipitated due to PEG fractionation and mostly remained in FBSsup, which retained the ability to support differentiation of preadipocytes (data not shown). Consistently, FBSppt supplementation did not cause any significant change in the differentiation of preadipocytes in FBS medium. However ABSppt supplementation significantly decreased preadipocyte differentiation in FBS medium, indicating the presence of an anti-adipogenic protein in ABSppt that is absent from FBSppt. Proteomic analysis identified two ABSppt-specific proteins, alpha-2-macroglobulin (A2M) and paraoxonase/arylesterase 1 (PON1), which are supposed to have an anti-adipogenic effect.

Alpha-2-macroglobulin (A2M) is an acute phase protein involved in host defense (16). This protein acts as a general protease inhibitor and has been implicated as a carrier protein for cytokines, growth factors, and hormones (17-19). It was shown that 3T3-L1 preadipocyte accumulates A2M from the culture medium, whereas the protein disappears during differentiation of preadipocytes (9). Further, the addition of A2M antibody to the culture medium abrogates the accumulation of A2M in preadipocytes, resulting in spontaneous adipocyte differentiation (9). Although the mechanism of differentiation inhibition by A2M has not been elucidated, it was suggested that A2M may inhibit a protease, such as calpain that is critically required for the expression of adipogenic genes (20, 21). Alternatively, an A2M fragment, generated by the cleavage of the full-length A2M by a protease was suggested to have an anti-adipogenic effect. In this study, two ABSppt-specific proteins were identified as A2M, but the molecular weight and pI values of these proteins appeared to be different from those of the full-length protein, as estimated by 2D-PAGE (Fig. 2 and Table 1). Interestingly, it was reported that the gene encoding for bovine A2M is alternatively spliced and produces the protein in various lengths (22). Therefore, we speculate that an A2M variant predominant in ABS and ABSppt may have a higher anti-adipogenic effect than the full-length protein.

Paraoxonase/arylesterase 1 (PON1) is expressed in the liver and secreted in the blood as an enzyme associated with high-density lipoprotein (HDL) (23). PON1 hydrolyzes organophosphates, warfare agents, and aromatic esters, which are unlikely physiological substrates. The enzyme also exhibits an antioxidant effect protecting against oxidation of lipoproteins by reactive oxygen species (ROS) (24, 25). ROS are generally known to cause cell damage and apoptosis, whereas they participate in normal cellular responses, including signal transduction pathways. Although the regulation of adipocyte differentiation by ROS has not been clearly elucidated, it was shown that insulin activates NADPH oxidase and increases intracellular ROS during differentiation of 3T3-L1 preadipocytes (26). In agreement with this result, the exposure of preadipocytes to low concentrations of exogenous ROS increases adipocyte differentiation (27). In addition, it was demonstrated that a PON1 variant, PON2, attenuates the accumulation of triglycerides in mouse macrophages by reducing intracellular oxidative stress and inhibiting diacylglycerol acyl transferase (11). These previous results explain another previous report in which serum PON1 activity was shown to be negatively related with obesity (12). Consistently, the results of this study showed that PON1, an ABS-specific protein, may have an anti-adipogenic effect by decreasing oxidative stress during the differentiation of preadipocytes.

In conclusion, the impaired differentiation of preadipocytes in ABS medium is, at least in part, due to the presence of an anti-adipogenic protein(s) in ABS. Although it is not yet clear which of the proteins is directly involved in suppression of adipocyte differentiation, we identified putative anti-adipogenic ABS proteins, A2M and PON1, which were previously shown to suppress differentiation of preadipocytes. Further investigations will clarify the current situation, which is ongoing in our group.

MATERIALS AND METHODS

Materials and general methods

All chemicals, insulin, porcine trypsin and soybean trypsin inhibitor were purchased from Sigma Aldrich, unless otherwise indicated. DMEM, calf serum, fetal bovine serum (FBS) and adult bovine serum (ABS) for cell cultures were from Hyclone. UV-Vis absorption was recorded using a Cary 100 UV-Vis spectrophotometer (Varion). Protein concentrations were de-

termined by Bradford assay (28-30).

Fractionation of serum proteins

Proteins in FBS and ABS were partially precipitated by the addition of polyethylene glycol 8000 (PEG, 10% (w/v)) to 10 ml of FBS or 10 ml of ABS. The protein precipitates, FBSppt and ABSppt, respectively, were collected by centrifugation at 28,000 g for 30 min and dissolved in 2.0 ml of PBS as 5x concentrated protein solutions. FBSppt and ABSppt solutions were then filter-sterilized and added to the medium for preadipocyte differentiation.

Cell culture and preadipocyte differentiation

Preadipocyte 3T3-L1 cells were maintained in DMEM containing 10% calf serum by incubation at 37°C in a humidified atmosphere of 5% CO_2. For differentiation of preadipocytes, cells were cultured in DMEM containing 10% FBS and grown to confluence. Preadipocyte differentiation was induced by changing the culture medium to differentiation medium (DMEM and 10% FBS or 10% ABS) containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/ml of insulin, and followed by incubation for 2 days. Cells were further incubated in differentiation medium containing 5 μg/ml of insulin for another 6 days by changing the medium every 3 days. In some experiments, the differentiation media were supplemented with the indicated concentrations of FBSppt or ABSppt at every medium change. The level of preadipocyte differentiation was evaluated by measuring the intracellular lipid content by oil red-O staining (31).

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent from 3T3-L1 cells after 5 days of differentiation. The cDNA was synthesized from 1 μg of RNA using a kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Amplification reactions were conducted using an ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with the following primers for the indicated adipogenesis marker genes: PPAR-γ (forward primer, 5′-AAGAGCTGACCCAATGGTTG-3′; reverse primer, 5′-ACCCTTGCATCCTTCACAAG-3′), CD36 (forward primer, 5′-TGGAGCTGTTATTGGTGCAG-3′; reverse primer, 5′-TGGGTTTTGCACATCAAAGA-3′), and FABP4 (forward primer, 5′-AGCCCAACTTGATCATCAGC-3′; reverse primer, 5′-ATGGTGGTCGACTTTCCATC-3′).

Determination of alpha-2-macroglobulin concentrations

Alpha-2-macroglobulin (A2M) in the serum and protein samples were quantified using trypsin binding and protection from trypsin inhibitor (32). Serum or protein samples containing A2M was incubated in 1 ml of 50 mM Tris-HCl pH 8.0 with 25 μg/ml of porcine trypsin at 37°C for 15 min, followed by further incubation for 15 min with 25 μg/ml of soybean trypsin inhibitor. Chromogenic trypsin substrate, 100 mM *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Sigma) was added to the mixture and incubated at 37°C for 30 min. The reaction was terminated by the addition of 100 μl of glacial acetic acid, after which the absorption at 410 nm was measured. Concentrations of A2M were calculated using a standard curve obtained with 0-40 μg/ml of commercial A2M (Roche Applied Science).

Enzyme assay for PON1

Arylesterase activity of PON1 was measured in 20 mM Tris-HCl pH 8.0, 1 M NaCl, and 1 mM $CaCl_2$ at room temperature. The reaction was initiated by addition of the substrate 1 mM phenylacetate, and the formation of the reaction product phenol was followed by measuring the absorption at 270 nm (ε270 nm = 1.31 mM^{-1} cm^{-1}) (10). Arylesterase activity was calculated from the initial slope of product formation and expressed in U of μmol/min^{-1} ml^{-1}. Paraoxonase activity of PON1 was measured by addition of the substrate 1 mM paraoxon, as described above, and the formation of the reaction product *p*-nitrophenol was followed by measuring the absorption at 412 nm (ε412 nm = 17.1 mM^{-1} cm^{-1}) (10). Paraoxonase activity was calculated from the initial slope of product formations and expressed in mU of nmol/min^{-1} ml^{-1}.

Statistical analysis

Data values were expressed as means ± SE. Differences in gene expression and intracellular lipid content were analyzed by an unpaired Student's *t*-test. A P value < 0.05 was considered statistically significant.

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