B cell activation factor (BAFF) is a novel adipokine that links obesity and inflammation

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Abstract

B cell activation factor (BAFF) is a novel member of the TNF ligand superfamily, mainly produced by myeloid cells. BAFF has been shown to participate in B-cell survival and B- and T-cell maturation. BAFF expression in adipocytes has been recently demonstrated. In the current study, we verified that BAFF expression is increased during adipocyte differentiation. BAFF expression was augmented by TNF-α treatment and was decreased by rosiglitazone treatment. BAFF secretion in lean and in *ob/ob* mice sera were compared and smaller amount of BAFF was secreted in *ob/ob* mice. mRNA and protein expression were different between epididymal and visceral adipose tissue. BAFF expression was also increased in *ob/ob* mouse adipose tissue. We sought to identify known BAFF receptors (BAFF-R, BCMA, and TACI) in adipocytes, and determined that all three were present and upregulated during adipocyte differentiation. However, the ex-pression of TACI was distinct from that of BAFF-R and BCMA under TNF-α and BAFF ligand treatment. BAFF-R and BCMA expression levels were upregulated under pro-inflammatory conditions, but TACI was reduced. Conversely, BAFF-R and BCMA expression levels were downregulated by rosiglitazone treatment, but TACI was increased. Taken together, our results suggest that BAFF may be a new adipokine, representing a link between obesity and inflammation.

Keywords: adipokine; B-cell maturation antigen; obesity; rosiglitazone; TNFRSF13C protein, human; TNFSF13B protein, human; transmembrane activator and CAML Interactor protein

Introduction

B cell activation factor (BAFF) was recently introduced as a novel member of the TNF ligand superfamily and is also named BLyS, THANK, TALL-1, and TNFSF13B (Moore *et al.*, 1999; Nardelli *et al.*, 2001). BAFF is a type II transmembrane protein, containing 285 amino acids. The soluble form of BAFF, cleaved by "furin-like" protease, is a 152 amino acid protein, with a mass of 17,038 daltons (Moore *et al.*, 1999). BAFF is produced by myeloid lineage cells, malignant B cells, activated T cells, and bone marrow stromal cells (Nardelli *et al.*, 2001; Novak *et al.*, 2002; Scapini *et al.*, 2003; Lavie *et al.*, 2004). The recombinant soluble BAFF (aa 134-285) binds specifically to human primary B cells and B cell tumor lines, but not to T cells, monocytes, NK cells, or granulocytes (Moore *et al.*, 1999).

BAFF has three receptors that belong to the TNF receptor superfamily: B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BAFF receptor (BAFF-R) (Gross *et al.*, 2000; Thompson *et al.*, 2001). These receptors are primarily expressed in B cells, but TACI and BAFF-R are known to be expressed by T cell subsets as well (Huard *et al.*, 2001; Ng *et al.*, 2004). Because of the receptor distribution, the studies on BAFF have been focused on B cells and T cells. It has been shown that BAFF is responsible for B cell survival and maturation (Mackey *et al.*, 1999; Craxton *et al.*, 2003), and also functions as a T-cell co-stimulatory molecule (Huard *et al.*, 2001; Ng *et al.*, 2004). Furthermore, overexpre-

ssion of BAFF results in autoimmune-like manifestations (Mackey *et al.*, 1999), and BAFF levels are elevated in human autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple myeloma (Zhang *et al.*, 2001; Novak *et al.*, 2004; Seyler *et al.*, 2005). The expression and functional effects of BAFF on other cell lineages have not been elucidated.

Adipose tissue has long been known to provide energy storage for the body, but recent studies have resulted in redefinition of this tissue as an endocrine organ (Mohamed-Ali *et al.*, 1998). Adipose tissue causes inflammation by secreting cytokines (such as TNF-α and IL-6), chemokines (CCL2; MCP-1), and acute-phase molecules (CRP, haptoglobin, SAA, PAI-1) (Fantuzzi *et al.*, 2005; Kim *et al.*, 2006). Moreover, the adipokines, specific cytokines that are mainly produced by adipocytes e.g. leptin, adiponectin, and resistin, are increasingly being shown to have important roles as mediators linking the adipose tissue and inflammation (Fantuzzi *et al.*, 2005; Kim *et al.*, 2006; Tilg *et al.*, 2006). Obesity, now considered to be a low-grade chronic inflammation state, increases the risk of many metabolic diseases such as type 2 diabetes, atherosclerosis, certain cancers, and autoimmune diseases (Wisse *et al.*, 2004; Fantuzzi *et al.*, 2005; Kim *et al.*, 2006; Tilg *et al.*, 2006). The dysregulation of adipokine secretion is often observed in patients with obesity; therefore, adipokines may provide a link between obesity and metabolic disorders. In this regard, further research into the identification and characterization of novel adipokines will improve our understanding of obesity and related diseases.

Previously, we have investigated the expression profiles of inflammatory genes in human SGBS adipocytes using DNA microarrays, and found that TNFSF13B (another term for BAFF) is also expressed by human adipocytes. We reported that TNFST13B expression is increased during adipocyte differentiation and TNF-α treatment (Do *et al.*, 2006). Recently, Pelekanou and his coworkers have reported the expression of BAFF in breast tumor lesions and also in adipocytes surrounding the tumor lesions (Pelekanou *et al.*, 2008). We were therefore interested in further examination of BAFF expression in adipocytes.

In adipocytes, secreted TNF acts on cells via an autocrine/paracrine pathway through TNFR1 activation (Nguyen *et al.*, 2005). Additionally, it has been reported that BAFF and BAFF receptors are co-expressed by B cells, and that BAFF may signal through an autocrine pathway as a member of the TNF ligand superfamily (Haiat *et al.*, 2006). Therefore, verification of the existence of BAFF receptors on adipocytes may lead to a greater understanding of the function of BAFF in adipocytes.

Here we provide the first report of the expression of BAFF and BAFF receptors in 3T3-L1 adipocytes. We examined mRNA and protein expression levels of BAFF during adipocyte differentiation, and their changes under pro-inflammatory and anti-inflammatory conditions. We also examined the existence of BAFF receptors on 3T3-L1 adipocytes and observed their changes in response to induction or inhibition of inflammation. BAFF and BAFF receptor expression in adipose tissue of *ob/ob* transgenic
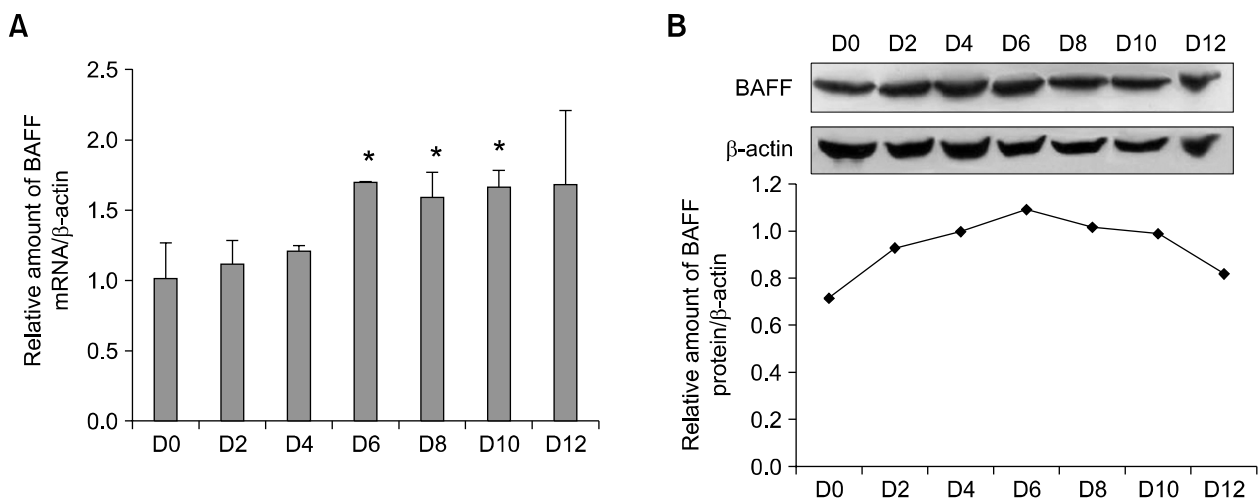

Figure 1. BAFF expression during adipocyte differentiation. (A) The relative amount of BAFF gene expression was evaluated by real time RT-PCR on every other day of 3T3-L1 adipocyte differentiation (*$P < 0.05$). (B) The relative amount of BAFF protein was evaluated by western blot analysis on every other day of 3T3-L1 adipocyte differentiation. Data are expressed relative to untreated control cells and represent means $\pm$ S.E. Experiments were conducted twice, each included triplication.

mice was examined as well. We further investigated the autocrine effects on 3T3-L1 adipocytes by treatment with soluble BAFF protein and examined the changes of BAFF receptors expression.

Results

BAFF expression is increased during adipocyte differentiation

In order to determine whether BAFF expression is affected by adipocyte differentiation, we collected 3T3-L1 adipocytes on every other day during the progression of differentiation. We examined mRNA and protein expression levels of BAFF using real time RT-PCR and western blot analysis, respectively. BAFF mRNA expression levels were increased as differentiation progressed, and were augmented 1.7-fold after six days of differentiation (Figure 1A). Similarly, protein expression levels were increased in accordance with differentiation, and on day six of differentiation BAFF protein level was 1.5-fold higher than before the induction of differentiation (Figure 1B).
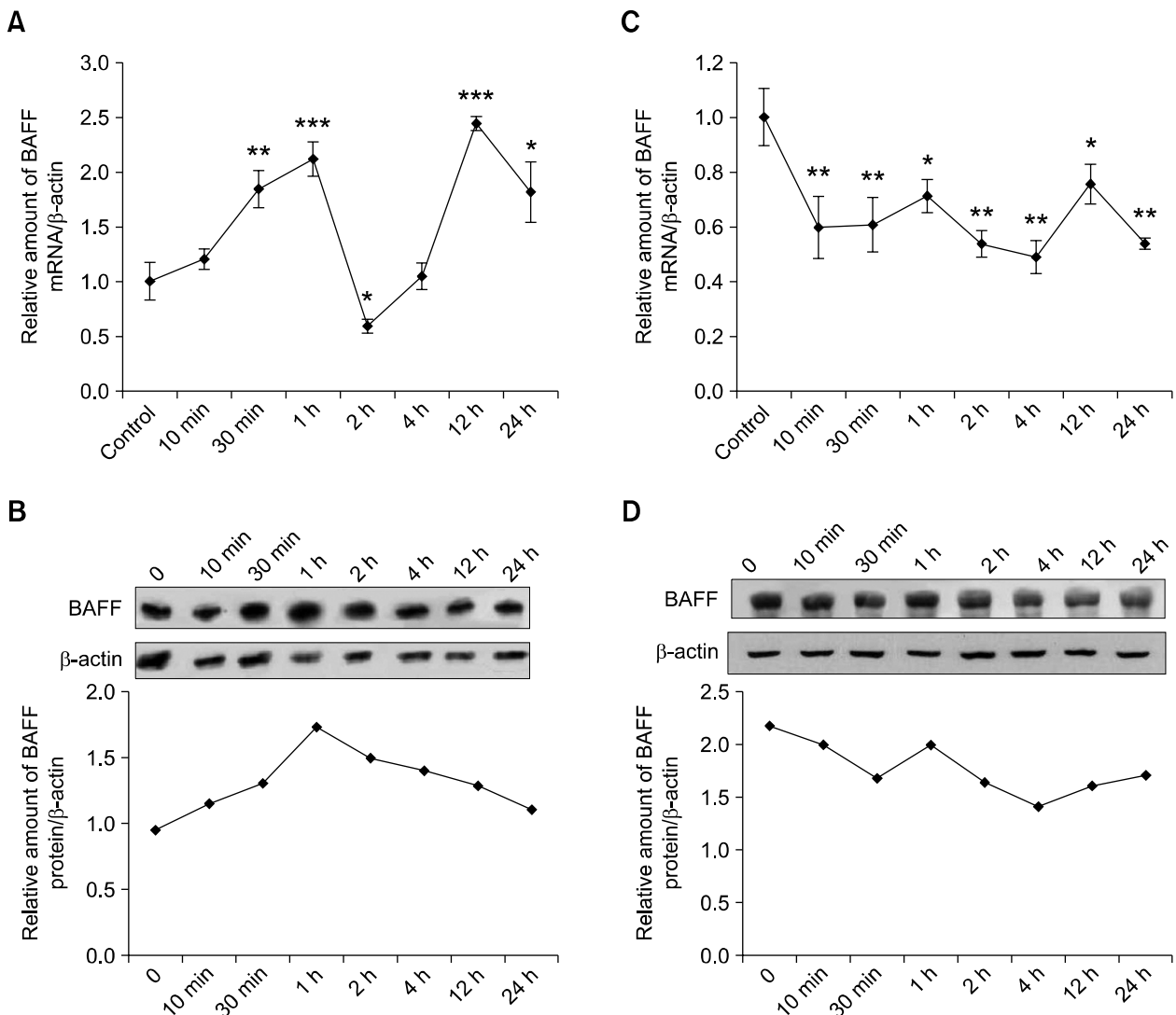

Figure 2. Changes of BAFF expression by TNF-α and Rosiglitazone treatment. (A) The relative amount of BAFF gene expression was evaluated by real time RT-PCR at different time points after simulation with 10 ng/ml TNF-α (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (B) The relative amount of BAFF protein was evaluated by western blot analysis at different time points after simulation with 10 ng/ml TNF-α. (C) The relative amount BAFF gene expression was evaluated by real time RT-PCR at different time points after simulation with 1.5 μM Rosiglitazone (*$P < 0.05$, **$P < 0.01$). (D) The relative amount of BAFF protein was evaluated by western blot analysis at different time points after simulation with 1.5 μM Rosiglitazone. Data are expressed relative to untreated control cells and represent means $\pm$ S.E. Experiments were conducted twice, each included triplication.

Changes of BAFF expression under pro- and anti-inflammatory conditions

In order to know whether BAFF expression is altered under inflammatory conditions, we applied TNF-α to fully differentiated 3T3-L1 adipocytes at a concentration of 10 ng/ml for 10 min, 30 min, 1 h, 2 h, 4 h, 12 h, and 24 h. We isolated mRNA and protein from adipocytes and measured BAFF expression levels using real time RT-PCR and western blot analysis. The overall expression of BAFF mRNA was upregulated by TNF-α treatment. Although the expression was diminished after 2-4 h of treatment, it was greatly increased by long-term treatment with TNF-α (Figure 2A). As Figure 2B shows, BAFF protein expression was slightly increased as well. The expression peaked after 1 h of treatment and then it steadily decreased, however the overall expression was increased from 20% to 80% by TNF-α.
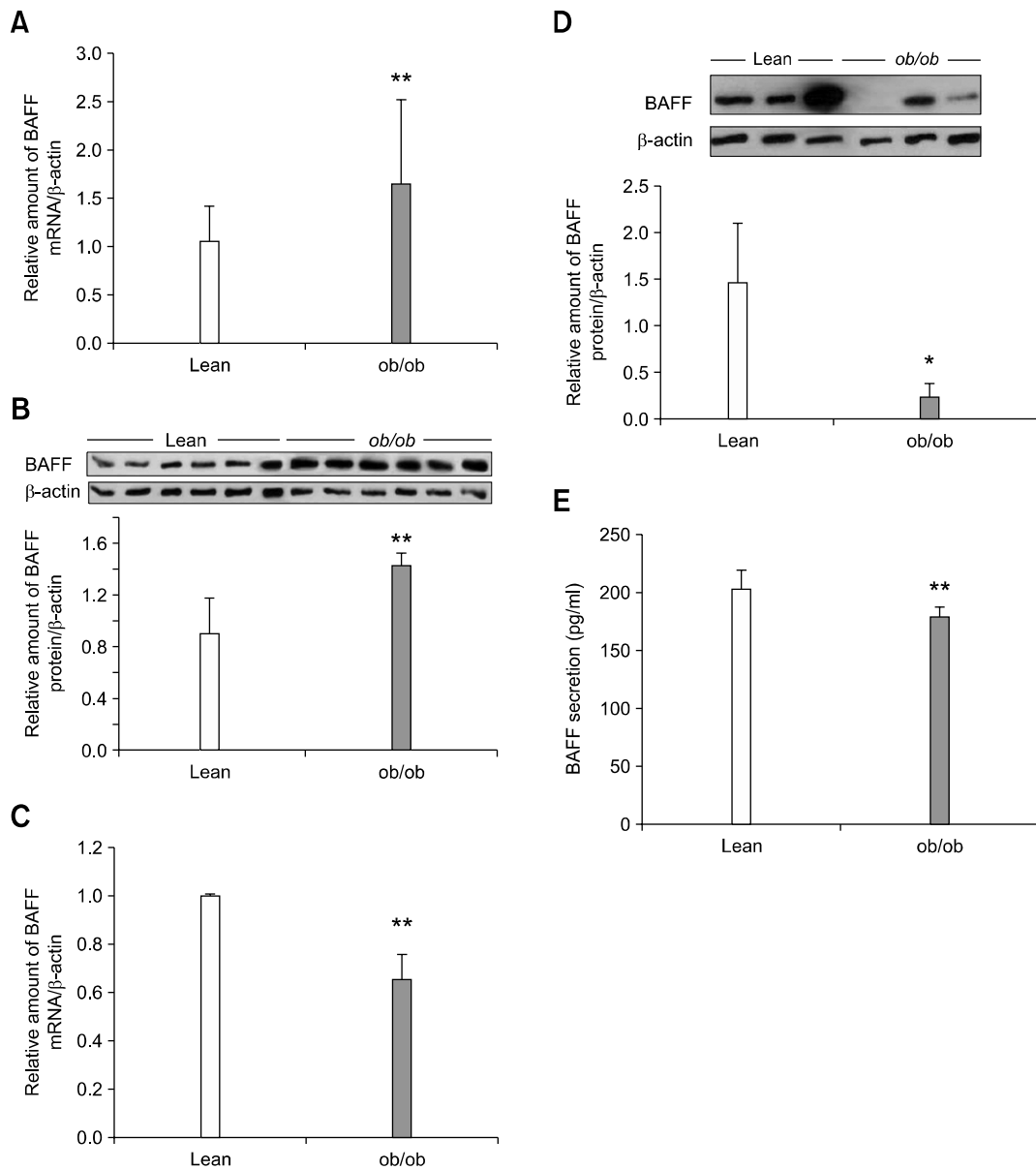
Furthermore, in order to investigate changes in

Figure 3. BAFF expression in *ob/ob* mice. The relative amount of (A) BAFF mRNA in EAT by real time RT-PCR, (B) BAFF protein in EAT by western blot analysis, (C) BAFF mRNA in VAT by real time RT-PCR, and (D) BAFF protein in VAT by western blot analysis were evaluated in lean and *ob/ob* transgenic mice. (E) BAFF protein levels in mouse serum detected by ELISA. Data are expressed relative to lean mice and represent means ± S.E. Experiments were conducted twice, each included triplication (n = 6 per group, *P < 0.05, **P < 0.01).

BAFF expression after inhibition of inflammation, we treated 3T3-L1 adipocytes with 1.5 μM of Rosiglitazone. We treated the fully differentiated adipocytes with Rosiglitazone for 10 min, 30 min, 1 h, 2 h, 4 h, 12 h, and 24 h. We isolated mRNA and protein from these adipocytes and BAFF expression was examined using real time RT-PCR and western blot analysis. BAFF mRNA expression was reduced roughly 30-45% under anti-inflammatory conditions (Figure 2C). BAFF protein expression was decreased by Rosiglitazone treatment as Figure 2D shows.

BAFF expression and secretion in *ob/ob* transgenic mice

BAFF expression appears to be upregulated during adipocyte differentiation and under induced inflammation. We also investigated BAFF expression in an animal model. We dissected epididymal adipose tissue (EAT) from lean control mice and *ob/ob* transgenic mice, and extracted total mRNA and protein. We then examined BAFF expression using real time RT-PCR and western blot analysis. Consistent with our hypothesis, we found that BAFF mRNA expression was increased 1.6-fold in obese mice (Figure 3A, $n = 6$), as was the protein expression level (Figure 3B, $n = 6$). In visceral adipose tissue (VAT), however, mRNA and protein expression are larger in lean than obese (Figure 3C, D). Secreted BAFF levels in mouse sera were quantified by ELISA and smaller amount of BAFF was detected in *ob/ob* mice sera (Figure 3E).

Expression of BAFF receptors in 3T3-L1 adipocytes

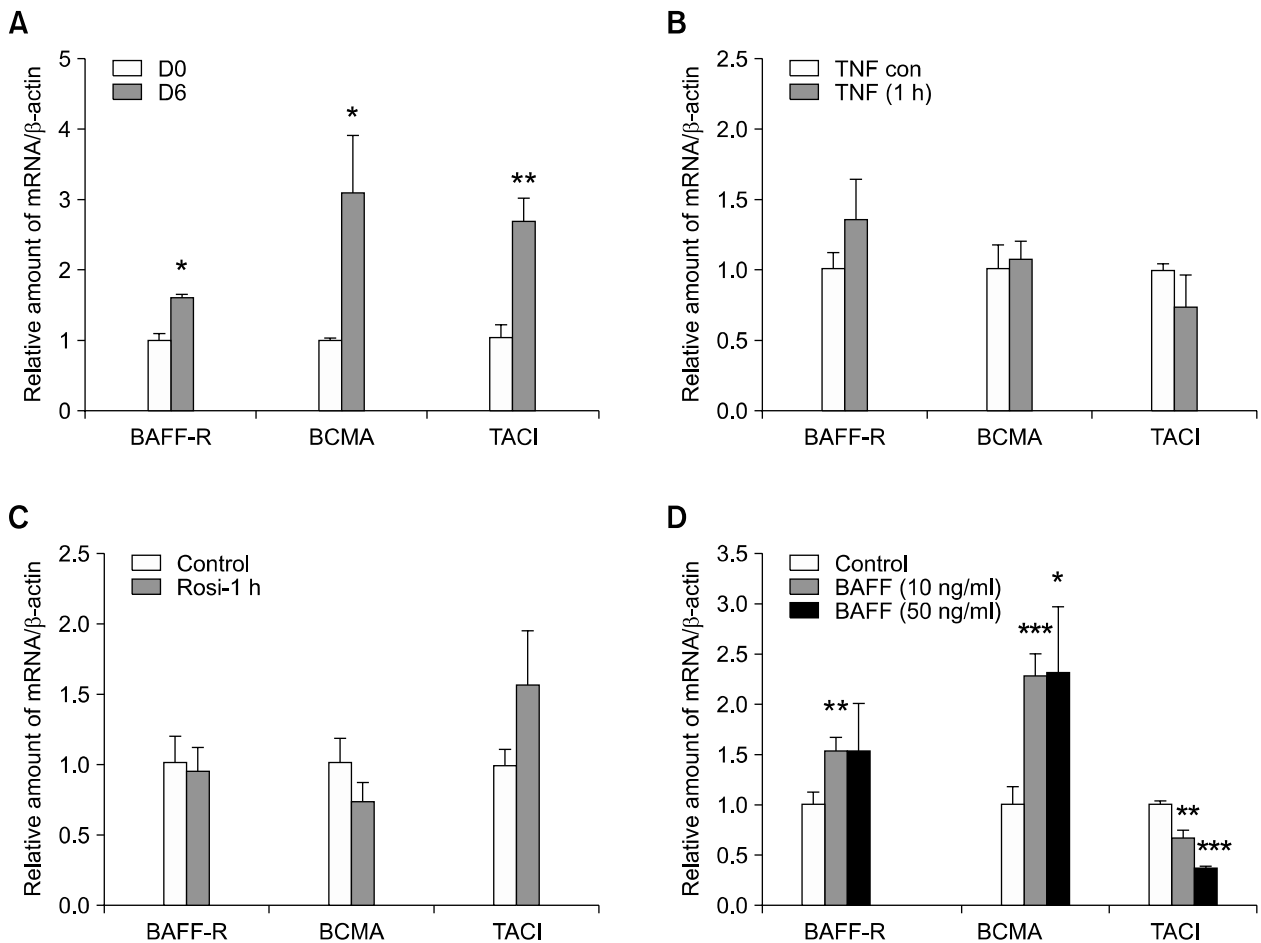As a ligand, BAFF signals through three different membrane-bound receptors: BAFF-R, BCMA, and

Figure 4. Changes of gene expression levels for BAFF receptors. The relative amounts of gene expression for BAFF receptors were evaluated by real time RT-PCR (A) in pre-adipocyte and fully differentiated adipocytes, (B) after 1 h TNF-α treatment, and (C) after 1 h Rosiglitazone treatment of fully differentiated adipocytes, and (D) after treatment with two different concentrations of recombinant BAFF. Data are expressed relative to untreated control cells and lean mice and represent means ± S.E. Experiments were conducted twice, each included triplication (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

TACI. We sought to investigate the presence of BAFF receptors in 3T3-L1 adipocytes. First, we examined whether these three receptors are expressed in 3T3-L1 adipocytes using real time RT-PCR. We found that all three receptors are present in adipocytes.

We further verified the changes in expression of these receptors during adipocyte differentiation. Specifically, we assessed expression of the BAFF receptors on day six of differentiation, under inflammatory and anti-inflammatory conditions produced by TNF-α or Rosiglitazone treatment. Figure 4A shows that expression of BAFF-R, BCMA, and TACI was increased 1.6-fold, 3-fold, and 2.7-fold, respectively, during adipocyte differentiation. After the induction of pro- and anti-inflammatory conditions, expression of the BAFF receptors was examined. According to Figure 2, one hour of treatment was most effective for both TNF-α and Rosiglitazone. We therefore chose the sample treated for one hour for analysis of gene expression levels by real time RT-PCR. As Figure 4B indicates, BAFF-R and BCMA expression levels were upregulated under pro-inflammatory conditions, but TACI was reduced. Conversely, BAFF-R and BCMA expression levels were downregulated by Rosiglitazone treatment, but TACI was increased (Figure 4C).

Autocrine signaling of BAFF in 3T3-L1 adipocytes

In order to investigate autocrine signaling of BAFF in adipocytes, we treated fully differentiated 3T3-L1 adipocytes with two different concentrations of recombinant BAFF (10 ng/ml and 50 ng/ml) for 24 h. We examined changes in mRNA expression levels of BAFF receptors to determine which receptors are dominantly activated by BAFF. As Figure 4D shows, BAFF-R and BCMA expression levels are upregulated 1.5-fold and 2.3-fold respectively by TNFSF13B treatment. Conversely, TACI expression is downregulated.

Discussion

We previously reported the expression of inflammatory molecules in SGBS human adipocytes, including BAFF. BAFF was initially identified in B cells and was thought mainly to be secreted from myeloid cells. It was subsequently shown that BAFF is also expressed in adipocytes. We therefore sought to determine its expression pattern, and examine its role as a link between obesity and inflammation. In the current study, we verified the expression of BAFF in adipocytes and examined the changes in BAFF expression that occur under inflammatory and anti-inflammatory conditions.

During adipocytes differentiation, BAFF expression level was 1.5-fold augmented. Although it is not dramatically increased, its expression level was 2-fold more increased by inducing inflammation. BAFF mRNA expression was significantly increased by short-term stimulation with TNF-α, but it was diminished after 2 h of treatment and again increased by long-term stimulation.

We used Rosiglitazone as an anti-inflammatory drug because it is a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist which has anti-diabetic properties and potential anti-inflammatory effects as recent studies have shown (Sánchez-Hidalgo *et al.*, 2005; Koufany *et al.*, 2008). Currently, Kolak and her collogues have reported that chronic Rosiglitazone therapy altered gene expression involved in fatty acid synthesis and inflammation (Kolak *et al.*, 2007). It indicates Rosiglitazone potentially affects both obesity and inflammation.

TNF-α signals through the autocrine loop, so the presence of BAFF receptors on adipocytes suggests an autocrine mechanism for BAFF. Thus, we treated differentiated adipoctyes with BAFF protein and found a reciprocal relationship in the expression patterns of TACI and BAFF-R and BCMA. While BAFF-R and BCMA expression was upregulated by the autocrine action of BAFF, TACI expression was downregulated. This expression pattern was the same under conditions of TNF-α stimulation.

BAFF-R is a principal receptor required for BAFF-mediated mature B cell survival (Thompson *et al.*, 2001), and it signals anti-apoptosis via NFκB and Bcl-2 (Langat *et al.*, 2008). TACI signaling activates NF-AT, AP-1, and NFκB (Gross *et al.*, 2000). Recently, Sakurai reported that BAFF changes its receptor preference kinetically (Sakurai *et al.*, 2007). In the early stage of B cell activation, BAFF preferentially transmits the signal via BAFF-R. Subsequently, BAFF-R signaling upregulates TACI expression on the cell surface and TACI provides negative feedback against excess B cell activation. Therefore, it is possible that TACI may also deliver a negative signal in adipocytes.

We got several interesting results from *in vivo* tests. BAFF mRNA and protein expression in EAT is greater in *ob/ob* mice, but it is reversed in VAT. It was interesting to see depot-specific differential expression of BAFF, but it is hard to make an explanation for it at this point. Instead, we have a need for further studies on cellular source of BAFF in different adipose tissues after fractionation into adipocytes and stromal/vascular cells since macrophages are infiltrated into adipose tissue in conse-

quence of obesity (Weisberg *et al.*, 2003). More-over, in measurement of BAFF serum levels in lean and *ob/ob* mice, BAFF secretion was surprisingly decreased in *ob/ob* mice which are resistant to the induction of autoimmune diseases. Autoimmunity in obesity is mainly mediated by leptin (La Cava *et al.*, 2004). According to La Cava, leptin, which is produced by adipocytes that are present in the perilymphonodal adipose tissue, promotes the differentiation of T_H1 cells and the secretion of pro-inflammatory cytokines (e.g. IFN-γ, TNF) while further inducing autoimmune diseases. In leptin-deficient *ob/ob* mice, increased production of T_H2-type cytokines (e.g. IL-4, IL-10) and reduced secretion of T_H1-type cytokines were observed. These mice were resistant to the induction of autoimmune diseases (La Cava *et al.*, 2004). It is very meaningful since overexpression of BAFF results in autoimmune-like manifestations (Mackey *et al.*, 1999). The absence of leptin possibly has an inhibitory effect on BAFF secretion and accordingly resists autoimmunity. At the same time, soluble form of BAFF is secreted after membrane-bound form of BAFF is cleaved by protease at Golgi apparatus (Moore *et al.*, 1999). Therefore, distribution of BAFF in cell and its condition for cleavage should be explored later. The BAFF expression and secretion level in diet-induced obesity should be thoroughly investigated in order to know a relationship between BAFF and autoimmune diseases.

In order to understand the role of BAFF as a new adipokine in relation to inflammation, a de-tailed study is needed. It would be interesting to block the expression of BAFF in adipocytes using siRNA, in order to study the significance of BAFF expression in adipocytes. Moreover, through stu-dying ligand and receptor binding, we may be able to identify which receptor is solely responsible for BAFF signaling in adipocytes.

Taken together, our study shows the possibility that BAFF is a novel adipokine which may be a link between obesity and inflammation. This is the first report presenting the expression of BAFF in 3T3-L1 adipocytes.

Methods

Materials

DMEM, high-glucose DMEM, BCS, FBS, were purchased from Hyclon (Logan, UT). Trypin/EDTA, insulin, dexame-thasone, and IBMX were purchased from Sigma-Aldrich (St. Louis, MO). Trizol for RNA extraction and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). 2× SYBR Green Mix was purchased from AB Applied Bioscience (Foster City, CA). Primers for the PCR analysis were purchased from Bioneer (Daejeon,

Korea). Antibody against BAFF was purchased from R&D Systems (Minneapolis, MN). Antibody against β-actin, anti-rat HRP-conjugated IgG, and anti-rabbit HRP-conjugated IgG were purchased from Cell Signaling Technology (Danvers, MA). Recombinant mouse TNF-α was purcha-sed from Sigma-Aldrich (St. Louis, MO). Recombinant mouse BAFF was purchased from R&D Systems (Minneapolis, MN). Rosiglitazone (Glaxo Smithkline, Brent-ford, MDX, UK) was kindly provided by Molecular Neuro-physiology Lab., POSTECH, Korea.

Cell culture and induction of adipocyte differentiation

3T3-L1 pre-adipocytes purchased from ATCC (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% BCS at 37°C in a humidified atmo-sphere of 5% CO_2. After 3 or 4 days, cells were collected after reaching 90% confluence by 0.05% Trypsin/0.53 mM EDTA treatment. After centrifugation (1,300 rpm, 5 min), cells were plated into 6 well plates at a density of 3 × 10^4 cell/well. At one day post-confluence (designated "day 0"), cell differentiation was induced with a mixture of 0.5 mM of methylisobutylxanthine, 0.25 mM of dexamethasone, and 5 mg/ml of insulin in DMEM containing 10% FBS. On day two and thereafter, DMEM supplemented with 10% FBS and 5 mg/ml insulin was subsequently replaced every two days. 3T3-L1 adipocytes, seven to eight days post-diffe-rentiation, were treated with 10 ng/ml TNF-α or 1 μM Rosiglitazone for 10 min, 30 min, 1 h, 2 h, 4 h, 12 h, and 24 h. Cells were exposed to 10 ng/ml or 50 ng/ml recom-binant BAFF for 24 h.

Mice

Six-week-old *ob/ob* transgenic mice and lean C57BL/6 mice were purchased from Central Lab. Animal Inc. (Seoul, Korea). Mice were fed standard chow diet until age eight wk. All mice were housed in cages on a 12-h day, 12-h night cycle, temperature 22 ± 1°C, humidity 55 ± 5%. At the time of the experiments, mice were killed under anes-thesia. Blood was collected in EDTA coated tubes and separated serum by centrifugation (2,000 *g*, 20 min). Epididymal adipose tissues were collected by dissection, weighed, quickly frozen by immediate immersion in liquid nitrogen and stored at -70°C for RNA and protein isolation.

Quantification of gene expression levels by real time RT-PCR

Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA). Cells were homogenized by passing four to five times through a 23 gauge needle. After approximately five min, samples were centrifuged for ten min at 4°C, and 12,000 rpm. Supe-rnatants were treated with chloroform and were mixed by vortexing for 15 s. After centrifugation, the colorless frac-tions were transferred into new tubes. For precipitation of total RNA, samples were mixed with isopropyl alcohol. The RNA pellet was washed with 75% ethanol and dissolved in RNase free water. RNA quantification was measured by determining the absorbance at 260 nm in a spectrophoto-meter (A_{260}). RNA samples were stored in a -70°C

deep-freezer. To prepare cDNA, total extracted RNA (1 μg) was mixed with oligo dT primer and RNase free water. Samples were incubated in a thermocycler to denature the RNA and anneal the oligo dT primer. The reaction was mixed with $5 \times$ first strand buffer, 20 mM of DTT, 10 mM of dNTP mix, RNase free water and Superscript II reverse transcriptase. Mixtures were incubated in a thermocycler (42°C for 1 h and 72°C for 7 min). Real time RT-PCR analysis was performed by Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, Foster City, CA). Samples were mixed with $2 \times$ SYBR Green Mix (Applied Biosystems, Foster City, CA), 0.5 μM of the appropriate primers, and cDNA, and were incubated in the Applied Biosystems 7500 Real Time PCR system for an initial denaturation at 94°C for 10 min, followed by 40 PCR cycles. Each cycle consisted of 95°C for 15 s, followed by 60°C for 1 min. To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 65°C for 15 s and heating slowly to 95°C with continuous measurement of fluorescence) were produced at the end of each PCR. Oligonucleotide primers were as follows: BAFF, 5'-GGCAGGTACTACGACCATCTC-3' and 5'-TGGGCCT-TTTCTCACAGAAGT-3'; BAFF-R, 5'-CCCCAGACACTTC-AGAAGGA-3' and 5'-AGGTAGGAGCTGAGGCATGA-3'; BCMA, 5'-ATCTTCTTGGGGCTGACCTT-3' and 5'-CTTT-GAGGCTGGTCCTTCAG-3'; TACI, 5'-GTGTGGCCACTT-CTGTGAGA-3' and 5'-CTGGTGCCTTCCTGAGTTGT-3'; and β-actin, 5'-AGCCATGTACGTAGCCATCC-3' and 5'-TCCCTCTCAGCTGTGGTGGTGAA-3'.

Quantification of protein expression levels by western blot analysis

After experimental treatment, cells were washed twice with ice-cold PBS and lysed with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium ortho-vanadate, 100 mM NaF, 1 mM PMSF and protease inhibitor cocktail, and stayed on ice for 30 min. Insoluble materials were removed by centrifugation at 15,000 g for 10 min at 4°C. The total concentration of extracted proteins was determined using the Bradford method. Total protein from dissected adipose tissue was isolated using homogenization buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM PMSF). Tissue was homogenized in 10 volumes of ice-cold homogenization buffer using glass homogenizer. After 10 min of incubation on ice, samples were centrifuged (12,000 rpm, 4°C, 20 min) and supernatants were subjected to quantification using the Bradford method. The proteins in the supernatants were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking for 1 h at room temperature with TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% nonfat milk, the membranes were incubated with the appropriate primary antibodies. To detect the antigen-bound antibodies, the blots were treated with secondary antibodies conjugated to HRP. Immuno-reactivity was detected using the ECL western blotting detection system.

Quantification of BAFF secretion in serum by ELISA

Measurement of BAFF secretion in lean and *ob/ob* mice serum is performed with mouse BAFF/BLyS/TNFSF13B immunoassay (R&D Systems, Quantikine) according to the manufacturer's instruction.

Statistical analysis

The data was analyzed by the ANOVA procedure of the Statistical Analysis System (SAS Institute, 1999-2001). Significant differences between groups were determined using Duncan's multiple range tests. Probability values of > 0.05 were considered statistically not significant.

Acknowledgements

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