Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 25 (2014) 1309–1316

Journal of Nutritional Biochemistry

Involvement of *de novo* ceramide synthesis in pro-inflammatory adipokine secretion and adipocyte–macrophage interaction☆

Yoji Hamada[a,*], Hiroshi Nagasaki[a,b], Atsushi Fujiya[c], Yusuke Seino[a], Qing-Long Shang[d,e], Takeshi Suzuki[d], Hiroyuki Hashimoto[d], Yutaka Oiso[c]

[a]Department of Metabolic Medicine, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
[b]Department of Physiology I, School of Medicine, Fujita Health University, 1-98 Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
[c]Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
[d]Pharmaceutical Research Laboratories, Sanwa Kagaku Kenkyusho Co, Ltd, 363 Shiosaki, Hokusei-cho, Inabe 511-0406, Japan
[e]Department of Microbiology, Harbin Medical University, Harbin 150086, China

Received 15 January 2014; received in revised form 14 May 2014; accepted 24 July 2014

Abstract

Interaction between adipocytes and macrophages has been suggested to play a central role in the pathogenesis of obesity. Ceramide, a sphingolipid *de novo* synthesized from palmitate, is known to stimulate pro-inflammatory cytokine secretion from multiple types of cells. To clarify whether *de novo* synthesized ceramide contributes to cytokine dysregulation in adipocytes and macrophages, we observed cytokine secretion in mature 3T3-L1 adipocytes (L1) and RAW264.7 macrophages (RAW) cultured alone or co-cultured under the suppression of *de novo* ceramide synthesis.

Palmitate enhanced ceramide accumulation and stimulated the expression and secretion of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in L1. The suppression of serine-palmitoyl transferase, a rate-limiting enzyme of *de novo* ceramide synthesis, by myriocin or siRNA attenuated those palmitate-induced alterations, and a ceramide synthase inhibitor fumonisin B1 showed similar results. In contrast, the inhibitor of sphingosine kinase or a membrane-permeable ceramide analogue augmented the cytokine secretion. Myriocin effects on the palmitate-induced changes were not abrogated by toll-like receptor-4 blockade. Although palmitate stimulated RAW to secrete tumor necrosis factor-α (TNF-α), it did not significantly increase ceramide content, and neither myriocin nor fumonisin B1 attenuated the TNF-α hypersecretion. The co-culture of L1 with RAW markedly augmented IL-6 and MCP-1 levels in media. Myriocin or fumonisin B1 significantly lowered these cytokine levels and suppressed the gene expression of TNF-α and MCP-1 in RAW and of IL-6 and MCP-1 in L1.

In conclusion, *de novo* synthesized ceramide partially mediates the palmitate effects on pro-inflammatory adipokines and is possibly involved in the interaction with macrophages.

Keywords: Ceramide; Palmitate; Adipocytes; Macrophages; Cytokines; Serine-palmitoyl transferase

1. Introduction

Metabolic syndrome is a growing concern of global public health as a risk factor for cardiovascular diseases, yet basic mechanisms of the syndrome are still arguable. Among various hypotheses, the abnormal cytokine secretion from adipocytes accompanied by pro-inflammatory interaction with macrophages has become a central paradigm that accounts for pathophysiology of metabolic syndrome [1]. The regulatory mechanisms of adipokines, however, have not been fully elucidated.

Palmitate, one of the representative saturated fatty acids in human plasma and adipose tissues [2], has been related to the pathogenesis of metabolic syndrome and type 2 diabetes mellitus [3]. Palmitate is known to induce insulin resistance in muscles [4] and dysfunction in pancreatic β-cells [5,6]. Palmitate also augments the gene expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) in adipocytes or other types of cells [7–9] and tumor necrosis factor-α (TNF-α) in macrophages [10]. Of note, plasma free fatty acids (FFAs) were shown to reach higher concentrations in obese and diabetic patients than those in healthy individuals [11–13].

Palmitate is known as a precursor of ceramide, a family of sphingolipids that possesses a variety of biological activities. Ceramide is produced from palmitate via the *de novo* synthetic pathway by the activity of serine-palmitoyl transferase (SPT), as well as from sphingomyelin by sphingomyelinase [14], and has drawn attention as a pivotal player in the pathophysiology of insulin resistance, metabolic syndrome and type 2 diabetes [14–17]. Ceramide impairs insulin signals by activating ceramide-activated

☆ Grants: This study was supported by research grants from the Ministry of Health and Welfare of Japan, Mitsui Life Social Welfare Foundation and Aichi D.R.G. Foundation.

* Corresponding author. Department of Metabolic Medicine, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan. Tel./fax: +81-52-744-2206.
E-mail address: yhama@med.nagoya-u.ac.jp (Y. Hamada).

http://dx.doi.org/10.1016/j.jnutbio.2014.07.008
0955-2863/

protein phosphatase to dephosphorylate Akt/PKB [18,19] and affects other signal proteins such as protein kinase C [20–22]. TNF-α-induced insulin resistance, disturbed insulin secretion and pancreatic β-cell apoptosis have been also related to ceramide production [23–25].

Several studies have shown that a membrane-permeable ceramide analogue, *N*-acetylsphingosine (C2 ceramide), stimulates IL-6 secretion from fibroblast [26] and TNF-α secretion from macrophages [10]. The latest study has revealed that LDL ceramides stimulate IL-6 and TNF-α secretion from cultured macrophages [27]. These data indicate that the ceramides contribute to pro-inflammatory cytokine release as well and thus it may be hypothesized that *de novo* synthesized ceramide mediates the palmitate-induced cytokine alterations, although the palmitate effects have been attributed to toll-like receptor-4 (TLR-4) signaling pathways [28].

Present study aimed to clarify whether or not *de novo* ceramide synthesis is involved in the palmitate-induced pro-inflammatory cytokine secretion from adipocytes and macrophages as well as in the interaction between the adipocytes and macrophages. Here we report the significant contribution of the *de novo* synthesized ceramide to the palmitate stimulation of IL-6 and monocyte chemoattractant protein-1 (MCP-1) secretion from adipocytes and to the interaction between adipocytes and macrophages.

2. Methods and Materials

2.1. Materials

3T3-L1 cells were purchased from ATCC (Manassas, VA, USA) and the murine RAW264.7 macrophages (RAW) were provided by the RIKEN BioResource Center through the National BioResource Project of the NEXT, Japan. SiRNAs for serine-palmitoyl transferase long chain-1 (SPTLC-1) and for TLR-4 along with negative-control siRNA were obtained from Applied Biosystems (Carlsbad, CA, USA). Anti-SPTLC-1 antibody was from ABGENT (San Diego, CA, USA). TLR-4 neutralizing antibody was obtained from Santa Cruz (Santa Cruz, CA, USA) and anti-TLR-4 antibody was from R&D Systems (Minneapolis, MN, USA). Myriocin, fumonisin B1 and C2 ceramide were purchased from BioMol (Philadelphia, PA, USA). C16 ceramide and cardiolipin were from Avanti Polar Lipids (Alabaster, AL, USA). Recombinant ceramide kinase was purchased from BPS Bioscience (San Diego, CA, USA). ELISA kits for IL-6, MCP-1, TNF-α, adiponectin and leptin were obtained from R&D Systems. Primers for SPTLC-1 and TLR-4 were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA) or Wako (Osaka, Japan).

2.2. Cell culture

3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) under 95% air and 5% CO_2 at 37°C. Two days after reaching full confluency, the cells were differentiated with 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone and 5 mg/ml insulin in DMEM containing 10% FBS for 2 days and then with 5 mg/ml insulin in the same medium for 2 days. Culture medium was then changed to DMEM with 10% FBS, and the cells were maintained for about two more weeks until experiments.

For co-culture experiments, mature 3T3-L1 adipocytes (L1) were cultured with RAW for 24 h using a transwell system (BD Falcon, Franklin Lakes, NJ, USA) as reported previously [29].

2.3. Palmitate stimulation of adipocytes under modulation of ceramide metabolism

L1 were cultured with 0–0.6 mM palmitate for 6 h to observe adipokine mRNA expression and for 24 h to examine the protein secretion to media. With 0.6 mM palmitate, 10 μmol/L myriocin (an SPT inhibitor), fumonisin B1 (a ceramide synthase inhibitor) or dimethylsphingosine (DMS) (a sphingosine kinase inhibitor) was added to modulate ceramide metabolism. Cells were also incubated with C2 ceramide, a cell-membrane-permeable ceramide analogue, at the concentration of 0.1–0.4 mM conjugated with bovine serum albumin at a 2:1 molar ratio. The resultant media and cells were collected and applied to measurement of adipokine protein levels and mRNA expression, respectively.

2.4. Knockdown of SPT

SiRNA for SPTLC-1 along with scrambled negative-control siRNA were transfected into 3T3-L1 adipocytes utilizing N-TER Nanoparticle siRNA Transfection System (Sigma). SiRNA–N-TER complex was formed following the manufacturer's instruction and mixed with serum-free DMEM at a final concentration of 80 nM. 3T3-L1 adipocytes in a 24-well plate were floated in the serum-free medium by trypsinization and incubated with 300 μl of the siRNA-contained medium for 2 h. Ten percent FBS was then added to the medium and incubated for additional 2 h, followed by 48 h-incubation with fresh DMEM containing 10% FBS. The cells were collected to assure mRNA reduction or were applied to further experiments of palmitate stimulation.

2.5. Suppression of TLR-4

To observe the involvement of TLR-4 in palmitate-induced adipokine secretion, L1 cells were incubated with 20 μg/ml TLR-4 neutralizing antibody for 2 h followed by stimulation with 0.6 mM palmitate for 24 h. Silencing of TLR-4 was also performed by treating the cells with siRNA for TLR-4.

2.6. Determination of adipokine proteins

Protein concentrations of IL-6, MCP-1, adiponectin, leptin and TNF-α were determined by sandwich ELISA kits (R&D Systems), following the manufacturer's procedures.

2.7. Real-time polymerase chain reaction for adipokines

Total RNA was extracted from cells using a HighPure RNA isolation kit (Roche, Mannheim, Germany) and reverse transcribed into cDNA by ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). Obtained cDNA was applied to real-time polymerase chain reaction. Adipokine mRNA was measured using the primer-probe sets of TaqMan Gene Expression Assays (Applied Biosystems) with a TATA box binding protein as a reference, following the manufacture's instruction. SPTLC-1 and TLR-4 mRNA were quantified using qPCR Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium) with 18S ribosomal RNA as a reference in M×3000 system (Stratagene, La Jolla, CA, USA). Primers used for SPTLC-1 were 5′-ggtgctggtggagatggt-3′ and 5′-ggattccttccaaaataagatgg-3′ and primers used for TLR-4 were 5′-ggactctgatcatggcactg-3′ and 5′-ctgatccatgcattggtaggt-3′, respectively.

2.8. Ceramide quantification

We have established a non-radioisotopic microplate assay for ceramide quantification, utilizing a sensitive ADP assay [30]. In brief, lipids were first extracted by Bligh–Dyer methods and the obtained extracts were evaporated. The samples were then dissolved by micellar formation with 20 μl of 10 mM imidazole buffer, pH 7, containing 7.5% β-octylglucoside, 5 mM cardiolipin and 0.2 mM diethylenetriamine pentaacetic acid by freeze–thaw cycling followed by sonication as previously described [31]. The micelle was mixed with 60 μl of 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, containing 20 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM dithiothreitol, 0.02% Tween 20 and 0.1 mg/ml bovine γ-globulins.

Kinase reaction was started by adding 10 μl of 5 mM ATP and 0.2 μg of recombinant ceramide kinase, an enzyme that demonstrated high substrate specificity to ceramide [32]. After 5 h of incubation at 37°C, the reactants were diluted by 2.5-fold to avoid interference of ATP and applied to ADP quantification utilizing an ADP Hunter Plus Assay (Amersham, Piscataway, NJ, USA) on a 96-well black microplate (BD Falcon). Resultant fluorescence was measured at excitation/emission wavelengths of 530/590 nm. This assay was validated for the measurement of up to 3000 pmol of ceramide by a linear standard curve with a small deviation (Fig. S1).

3. Results

3.1. Effects of palmitate on adipokine expression and secretion

We first examined mRNA expression of SPT and TLR-4 during differentiation of 3T3-L1 cells. Both SPT and TLR-4 expressions were increased toward the third week of differentiation (Fig. S2); thus, experiments were performed using cells 2–3 weeks after inducing differentiation. The differentiated L1 adipocytes at a resting state expressed and secreted abundant adiponectin, MCP-1 along with detectable levels of IL-6 and leptin, while TNF-α protein was under detection limit of the present assay and its mRNA expression was extremely lower than other cytokines (Table S1).

Palmitate increased the mRNA expression and protein secretion of IL-6 and MCP-1 in L1 in a dose-dependent fashion (Fig. 1). In contrast, neither the mRNA expression nor protein levels of adiponectin and leptin were affected by palmitate. Palmitate showed no significant effects on TNF-α mRNA expression, and its protein secretion from L1 remained undetectable after the palmitate stimulation (data are not shown).