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*J Immunol 2007; 179:4829-4839; *
doi: 10.4049/jimmunol.179.7.4829
http://www.jimmunol.org/content/179/7/4829

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Aging Up-Regulates Expression of Inflammatory Mediators in Mouse Adipose Tissue

Dayong Wu,†‡ Zhihong Ren,‡‡ Munkyong Pae,* Weimin Guo,* Xuelin Cui,* Alfred H. Merrill,§ and Simin Nikbin Meydani†‡

Obesity is a leading risk factor for type 2 diabetes (T2D). Aging is associated with an increase in T2D incidence, which is not totally explained by the much lower prevalence of obesity in the elderly. Low-grade inflammation in adipose tissue (AT) contributes to insulin resistance and T2D. Thus, we determined whether inflammatory responses are up-regulated with age in AT. The results showed that visceral AT from old C57BL mice had significantly higher mRNA expression of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and COX-2 and lower expression of anti-inflammatory PPAR-γ than those of young mice. We further showed that adipocytes (AD) and not stromal vascular cells including macrophages (Mφ) were the cells responsible for this higher inflammatory state of the aged AT, suggesting that the age-associated increase in AT inflammation is distinguished from that seen in obesity, in which Mφ are the main contributors. However, peritoneal Mφ of either age (young or old) produced more TNF-α and IL-6 after incubation in old AD-conditioned medium compared with young AD-conditioned medium. This suggests that in addition to producing more inflammatory cytokines, AD from old mice induce a higher inflammatory response in other cells. Sphingolipid ceramide was higher in old compared with young AD. Reducing ceramide levels or inhibiting NF-κB activation decreased cytokine production, whereas the addition of ceramide increased cytokine production in young AD to a level comparable to that seen in old AD, suggesting that ceramide-induced activation of NF-κB plays a key role in AT inflammation. The Journal of Immunology, 2007, 179: 4829–4839.

Received for publication October 6, 2006. Accepted for publication July 27, 2007.

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NF-κB is viewed as a central transcription factor in regulating inflammatory responses because it controls the activation of genes encoding synthesis of the majority of inflammatory markers and mediators including TNF-α, IL-6, IL-1β, IL-8, MCP-1, iNOS, cyclooxygenase (COX)-2, and adhesion molecules (16). Recent studies have pointed to the IκB kinase-β/NF-κB signaling pathway as a potential mediator in the cross-talk between inflammation and insulin resistance. There are many environmental and physiological factors that are known to affect NF-κB activation, and the sphingolipid ceramide is suggested to be one such activating agent (17–20). In accordance with these findings, a number of studies have suggested that ceramide may serve as an intermediate linking excess lipids and inflammatory cytokines to the induction of insulin resistance (21). Elevated levels of ceramide in muscle and liver have been reported in insulin-resistant rodents such as genetically obese Zucker rats (22) and transgenic mice with muscle- and liver-specific overexpression of lipoprotein lipase (23). Ceramide has been shown to mediate insulin resistance induced by TNF-α (24, 25) and palmitate (26, 27).

An increased inflammation state with aging, the so-called “inflamm-aging,” has been proposed mainly based on peripheral levels of inflammatory cytokines and acute phase reaction proteins (28–31). Because insulin resistance sharply increases with advancing age and low-grade inflammation in adipose tissue plays a key role in the development of insulin resistance, we hypothesized that aging is associated with increased adipose tissue inflammation. Furthermore, because both ceramide and NF-κB are implicated in insulin resistance in insulin target cells as well as in age-associated up-regulation of inflammatory responses (19, 32–40), we conducted experiments to determine the role of ceramide and NF-κB in age-associated adipose tissue inflammation.

Materials and Methods

Animals

Specific pathogen-free young (5–6 mo) and old (22–24 mo) male C57BL/6NIA mice were purchased from National Institute on Aging colonies at Harlan Sprague Dawley. Mice were individually housed in cages maintained at a constant temperature and humidity with a 12-h light/dark cycle. Mice were watered and fed nutritionally adequate autoclaved Teklad 7012 mouse chow (Harlan Teklad) ad libitum. All conditions and handling of the animals were approved by the Animal Care and Use Committee of the Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston, MA) and conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Tissue collection, RNA extraction, and real-time PCR

After mice were euthanized by CO₂ asphyxiation, visceral (epididymal and perirenal) and s.c. (inguinal) adipose tissues were collected into cryovials. Tissues were immediately put in liquid nitrogen and then stored at −80°C before RNA extraction. Adipose tissue was homogenized using a Tissue-Tearor (Biospec Products) and total RNA was extracted from homogenized tissues using TRIzol reagent (Invitrogen Life Technologies). RNA concentration was determined by measuring absorbance at 260 nm. All samples had a ratio of 260/280 nm at >1.8. RNA was reverse transcribed into cDNA using a A3500 kit (Promega) reverse transcription system, and the cDNA was used to conduct TaqMan real-time quantitative PCR in an ABI 7300 real-time PCR system (Applied Biosystems) following the manufacturer’s instruction. PCR conditions were at 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Primer and fluorescent probe sets were purchased from Applied Biosystems for the following: IL-1β (product no. Mm00434228_m1), IL-6 (product no. Mm00446190_m1), TNF-α (product no. Mm00443258_m1), COX-2 (product no. Mm00478374_m1), and peroxisome proliferator-activated receptor (PPAR)-γ (product no. Mm00449045_m1). Amplification with GAPDH primers (product no. 4352339E) was conducted on all samples. A critical threshold cycle (Ct) value was determined for each reaction and reflected the cycle number at which the DNA amplification was first detected. Relative transcript levels were calculated as $E = 2^{-ΔCt}$ (where $E$ is the gene expression value and $ΔCt$ is the difference in crossing points between GAPDH and genes).

Adipocytes and stromal vascular cell (SVC) isolation

Epididymal adipose tissue was collected under sterile conditions, weighed, and put in a container containing 1 ml of sterile PBS plus 2% BSA. Adipose tissue was minced into pieces of ~30 mg and incubated in 1 mg/ml collagenase type I (Worthington Biochemical) in a shaking water bath at 37°C for 1 h. Digested cells were passed through a 100-μm nylon cell strainer (BD Falcon) and then centrifuged at 450 × g for 10 min. The cells floating on the top were transferred to a new tube as adipocytes. The cell pellet (SVC) on the bottom was resuspended in 1 ml of RBC lysis buffer (Sigma-Aldrich) and incubated for 1 min at room temperature to remove RBC. Both adipocytes and SVC were washed twice with DMEM plus 10% FBS. After adipocytes and SVC were separately incubated overnight in DMEM plus 10% FBS to become quiescent, the old medium was removed and cells were washed using fresh medium before the experimental treatments. The condition for all cell incubations conducted in this study was at 37°C in 5% CO₂ and a 100% humidified atmosphere.

Immunohistochemistry of adipose tissue

Adipose tissue was fixed for 8 h in formalin fixative and embedded in paraffin. For immunostaining, the sections were deparaffinized with xylene, rehydrated through a series with ethanol, and incubated in 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Immunostaining was conducted using the Vectastain Elite ABC kit (Vector Laboratories). Nonspecific binding was blocked by incubation with normal rabbit serum for 30 min. Sections were stained with 1/100 diluted rat anti-mouse F4/80 Ab (Serotec) at 4°C overnight. Sections were then incubated with a biotinylated secondary Ab (anti-rat IgG) for 30 min, washed, and incubated for another 30 min with ABC (avidin and biotinylated enzyme complex) reagent. Color was developed by adding peroxidase substrate diaminobenzidine. Sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich) and, finally, mounting solution and coverslips were added.

FACS analysis for F4/80⁺ cells (Mφ) in adipose tissue

SVC isolated from epididymal adipose tissues were counted and aliquots of 1 × 10⁶ cells/sample were transferred to new tubes. Cells were centrifuged at 450 × g for 5 min and resuspended in FACS buffer (PBS plus 2% FBS). Cells were incubated on ice covered with a foil sheet for 30 min in Fc block (20 mg/ml purified rat anti-mouse CD16/CD32 mAb) (BD Pharmingen) and then for 45 min with alloxycyanin-conjugated anti-F4/80 Ab (5 μg/ml) (Caltag Laboratories) or rat anti-mouse isotype control Ab in 100 μl of FACS buffer. After incubation, 200 μl of FACS buffer was added to the cells, and the cells were then centrifuged at 250 × g for 5 min. Cells were resuspended in 1 ml of FACS buffer and washed twice. Cells were analyzed using a FACSCalibur cytometer, and data analysis was conducted using CellQuest software (BD Biosciences).

Peritoneal Mφ isolation and incubation with adipocyte-conditioned medium (ACM)

Peritoneal exudate cells were obtained by peritoneal lavage with cold Ca²⁺- and Mg²⁺-free HBSS (Invitrogen Life Technologies). Peritoneal exudate cells were enriched for Mφ using the method of Kumagai et al. (41). Briefly, peritoneal exudate cells were suspended in endotoxin-free RPMI 1640 (BioWhittaker) medium supplemented with 25 mM HEPES, 2 mM glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 2% FBS. The cells were plated on 24-well plates (Falcon Labware) and allowed to adhere for 2 h at 37°C. Nonadherent cells were removed by vigorous washing. Peritoneal exudate cells prepared in this manner were at least 90% Mφ. The percentage of Mφ that adhered to the plates did not differ with age (data not shown). To make ACM, equal amounts of quiescent adipocytes from young and old mice were incubated separately for 24 h and the resulting culture medium was collected as ACM and stored at −80°C. ACM from young or old mice was added to Mφ from young or old mice in a 2 × 2 combination and incubated for 24 h. The culture medium were collected for IL-6, TNF-α, and PGE2 analysis and Mφ were dissolved in 1 N NaOH for total cell protein analysis using the bicinchoninic acid protein assay kit (Pierce).
In the experiments on modulating cellular ceramide levels, cell-protein was measured using the bicinchoninic acid protein assay kit from Pierce. In the experiments on inhibiting NF-κB, IL-6, TNF-α, and COX-2, we first surface stained with allophycocyanin-conjugated anti-mouse F4/80 Ab (CalTag Laboratories). After a wash with FACS buffer, cells were fixed with an intracellular fixation buffer (eBioscience) containing 4% paraformaldehyde and then incubated with FITC-conjugated anti-mouse IL-6 and PE-conjugated anti-mouse TNF-α Abs (both from BD Pharmingen) in permeabilization buffer (eBioscience) for 30 min at room temperature. IL-6 and TNF-α levels in F4/80⁺ cells (Mφ) were analyzed by a FACSCalibur flow cytometer (BD Biosciences).

Sphingolipid analysis

The methods for sphingolipid analysis have been described in depth previously (43). Briefly, to each collected adipocyte sample in a glass test tube (0.5 ml of methanol, 0.25 ml of chloroform, and the internal standards were added. Samples were sonicated and incubated overnight at 48°C. After cooling, the glycerolipids were removed by adding 75 μl of 1 M KOH and incubating for 2 h at 37°C. After incubation, one fraction was collected for sphingoid bases and other highly polar compounds, and for the more complex lipids the extract was acidified with 3 μl of glacial acetic acid and the lipids were recovered in the organic phase after the addition of 1 ml of chloroform and 2 ml of water. After concentration and filtering, the samples were analyzed by liquid chromatography, electrospray tandem mass spectrometry on a PE Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source. The concentrations of sphingolipids in adipocytes were measured by total cellular DNA.

Statistical analysis

Data were analyzed using a SYSTAT statistical package (SYSTAT 10.0, 2000). The difference between two age groups was assessed using Student’s t test and the difference among treatments was assessed using Student’s paired t test followed by Bonferroni adjustment. Results were expressed as mean ± SE. Significance was set at p < 0.05.

Results

Adipose tissue mRNA expression of inflammatory molecules is up-regulated with aging

To determine whether adipose tissue exhibited the age-related increase in inflammatory molecules, we first determined the mRNA levels of classic markers of inflammation and found that epididymal adipose tissue from old lean mice have higher mRNA expression of IL-1β, IL-6, TNF-α, and COX-2 and lower mRNA expression of PPAR-γ, a nuclear receptor possessing anti-inflammatory and insulin-sensitizing properties, compared with those from young lean mice (Fig. 1). We also sampled mRNA expression of these molecules in different fat depots and found that the other depot of visceral fat, i.e., perirenal fat, as well as s.c. fat (inguinal adipose tissue) from old mice, also had a higher expression of inflammatory molecules compared with that of young mice. However, s.c. (inguinal) adipose tissue expressed much lower levels of mRNA for those molecules (data not shown). Because visceral

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Epididymal adipose tissue of old mice has higher mRNA expression of proinflammatory mediators compared with that of young mice. Epididymal adipose tissue was collected from young and old mice. Total RNA in adipose tissue was extracted and reverse transcription was conducted to generate single strand cDNA, which were further amplified by real-time PCR using specific primers for each inflammatory mediator. Quantification of mRNA was normalized with GAPDH. Results represent mean ± SE. n = 7. *p < 0.05 compared with young mice by Student’s t test.

### Table I. Macrophages (F4/80⁺ cells) present in epididymal adipose tissue

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Body weight (g)</th>
<th>AT weight (g)</th>
<th>Total SVC (×10⁶ cells)</th>
<th>Mδ/VSC (%)</th>
<th>Total Mδ (×10⁶ cells/g)</th>
<th>SVC/AT (×10⁶ cells/g)</th>
<th>Mδ/AT (×10⁶ cells/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>29.9 ± 0.86</td>
<td>0.88 ± 0.1</td>
<td>2.08 ± 0.44</td>
<td>32.28 ± 3.39</td>
<td>0.64 ± 0.17</td>
<td>2.23 ± 0.22</td>
<td>0.71 ± 0.1</td>
</tr>
<tr>
<td>Old</td>
<td>35.2 ± 0.45</td>
<td>1.24 ± 0.1</td>
<td>5.33 ± 0.89</td>
<td>18.22 ± 2.64</td>
<td>0.88 ± 0.17</td>
<td>4.14 ± 0.45</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td>Young vs old</td>
<td>p = 0.000</td>
<td>p = 0.017</td>
<td>p = 0.006</td>
<td>p = 0.007</td>
<td>p = 0.35</td>
<td>p = 0.002</td>
<td>p = 0.99</td>
</tr>
</tbody>
</table>

* Mean ± SE, n = 6–9 in young mice and n = 7–10 in old mice. AT, Adipose tissue.
adipose tissue had a higher expression of the mRNA of these molecules and has been shown to play a more prominent role in insulin resistance than s.c. adipose tissue (44, 45), we used only visceral (epididymal) adipose tissue in the subsequent experiments.

Number of MΦ in adipose tissue is not different between young and old mice

Recent studies reported a significant infiltration of MΦ in the adipose tissue of diet-induced or genetic mouse models of obesity. These MΦ have been shown to express higher levels of inflammatory genes and are responsible for almost all TNF-α, a significant amount of iNOS, and some of IL-6 expression in adipose tissue (11, 12). To determine whether the observed higher mRNA levels of the inflammatory molecules in old mice are also due to higher MΦ infiltration in old mice compared with young mice, we quantified the MΦ present in SVC and calculated the percentage of MΦ in visceral adipose tissue from young and old mice. As can be seen in Table I, the percentage of MΦ in SVC from old mice was about half (56%) that of young mice. However, old mice had almost twice (1.86-fold) as many SVC in the same amount of adipose tissue by weight compared with young mice, resulting in no difference in the number of MΦ per gram of adipose tissue between young and old mice (Table I). Consistent with this, the immunohistochemistry of adipose tissues from both young and old mice showed a comparable abundance of MΦ (F4/80+ cells) (Fig. 2). The characteristic ring-like MΦ clusters were scattered at a low frequency in both young and old mice, similar to what has been previously reported in lean mice (11, 12). Furthermore, there was no difference in MCP-1 production, a molecule responsible for MΦ infiltration into adipose tissue, between young and old mice (Fig. 3). Adipocytes and not nonadipocytes (SVC) in adipose tissue are the main contributors to the age-related up-regulation of inflammatory response

Because the higher expression of inflammatory molecules with age could not be explained by more infiltration of MΦ into the adipose tissue of old mice compared with that of young, we determined the production of inflammatory molecules by isolated SVC and adipocytes from young and old mice. Adipocytes were separated from SVC (containing MΦ) in the adipose tissue of young and old mice, and production of the representative inflammatory mediators IL-6, TNF-α, and PGE2 was measured. As shown in Fig. 4, adipocytes produced a significant amount of IL-6 and PGE2, which was further increased by LPS. TNF-α production, however, was detectable only in LPS-stimulated cells at levels lower than those of IL-6. More importantly, under both unstimulated (not including TNF-α) and LPS-stimulated conditions, adipocytes from old mice generated significantly more IL-6, TNF-α, and PGE2 compared with those from young mice (Fig. 4). In contrast, IL-6, TNF-α, and PGE2 production by SVC was not significantly different between young and old mice (Fig. 5). These results suggest that adipocytes play a key role in the higher expression of inflammatory molecules with age.

Environment of adipose tissue of old mice, compared with that in young mice, induced greater inflammatory response in MΦ

MΦ have been suggested as being major contributors to obesity-related inflammation in adipose tissue. It has been further suggested that, in obesity, adipocyte-initiated signals attract and activate MΦ to further propagate inflammation, which amplifies the
initial insulin resistance-inducing signal. We observed no difference in SVC production of inflammatory products. This, however, does not rule out the possibility that individual cellular components of SVC, i.e., Mφ/H9278, might exhibit age-related differences in the production of these products. Thus, we gated the Mφ/H9278 population in SVC to determine intracellular IL-6 and TNF-α levels and found that both were significantly higher in old compared with young mice (Fig. 6). Together with the observed lower percentage of Mφ/H9278 in the SVC of old compared with young mice, this result may explain why there was no age-related difference in the total SVC production of inflammatory products. Because age-related differences in IL-6 and TNF-α production are not generally observed in macrophages residing in other tissues such as peritoneal Mφ, we hypothesized that the environment in the adipose tissue of old mice may be responsible for promoting the inflammatory response of Mφ. To test this, we evaluated whether conditioned medium from adipocytes of young and old mice had a differential effect on the production of inflammatory molecules by Mφ. To obtain adequate purified Mφ for this experiment, peritoneal Mφ were used. Young or old peritoneal resident Mφ were cultured in conditioned medium from young or old adipocytes. IL-6 concentrations in conditioned medium from adipocytes were measured and subsequently subtracted from the IL-6 concentrations produced in cultures of Mφ stimulated with ACM. TNF-α was not detectable in conditioned medium. As shown in Fig. 7, ACM from old mice induced significantly higher production of IL-6 and TNF-α by Mφ from either young or old mice compared with that when young ACM was used. Similar results were observed with PGE2 production (data not shown). This effect of the ACM was not due to

**FIGURE 4.** Adipocytes from old mice produce more IL-6, TNF-α, and PGE2 than those from young mice. Epididymal adipose tissues were collected from young and old mice and adipocytes were isolated using collagenase digestion. Adipocytes were incubated for 24 h to become quiescent before being stimulated with LPS (0.2 μg/ml) for another 24 h. Culture medium was collected and analyzed for IL-6 and TNF-α using ELISA and PGE2 using RIA. Production of IL-6 (A), TNF-α (B), and PGE2 (C) was normalized with total cellular DNA. Results represent mean ± SE, n = 7–10; *, p < 0.05 and **, p < 0.01 by Student’s t test for the age difference.

**FIGURE 5.** IL-6, TNF-α, and PGE2 production is not significantly different between stromal vascular cells of young and old mice. Epididymal adipose tissues from young and old mice were digested using collagenase and SVC were isolated from adipocytes. RBC were removed using RBC lysis buffer. SVC were incubated for 24 h to become quiescent before being stimulated with LPS (0.2 μg/ml) for another 24 h. Supernatants were collected and analyzed for IL-6 and TNF-α using ELISA and PGE2 using RIA. Production of IL-6 (A), TNF-α (B), and PGE2 (C) was normalized with total cellular protein. Results represent mean ± SE, n = 6.
differences in cell death and the subsequent release of intracellular compounds between young and old adipocytes during the incubation time required to collect ACM. We determined cell viability by two different methods: 1) MTS, which is based on the conversion of MTS tetrazolium to formazan product by metabolically active cells and thus estimates the number of living cells; and 2) LDH, which is released in the medium when cells lose their integrity and thus is a reliable indicator of cell death. As shown in Fig. 8, there was no significant difference in either cell viability determined by MTS (panel A) or the percentage of LDH release (panel B) between young and old adipocytes. These results indicate that the observed age-associated difference in inflammatory molecule production mainly reflects functional changes with age. Taken together these data suggest that adipocytes are a key factor in age-associated up-regulation of the adipose tissue inflammatory state.

Ceramide is involved in age-associated up-regulation of IL-6 and TNF-α

Both lipid messenger ceramide (24, 26, 29, 46, 47) and inflammatory cytokine IL-6 (48–52) and TNF-α (53–57) have been shown to induce insulin resistance. Furthermore, ceramide activates transcription factors involved in the regulation of several inflammatory
molecules such as NF-κB (17–20) and AP-1 (58, 59). Thus, to determine the underlying mechanism of the age-related increase in adipocyte IL-6 and TNF-α production, we determined the cellular levels of ceramide and other related sphingolipids in the adipocytes of young and old mice. As can be seen in Fig. 9A, adipocytes from old mice have higher levels of ceramide as well as sphingomyelin, which can be hydrolyzed by sphingomyelinase (SMase) to produce ceramide, compared with that of young mice. Likewise, glucosylated ceramide was higher in old than in young adipocytes. Sphingosine, the deacylated product of ceramide and a major sphingolipid backbone, has been shown to have similar effects as ceramide in activating COX-2 (60). Recent studies have suggested that ceramide-1-phosphate (C1P) and sphingosin-1-phosphate (S1P), phosphorylated products of ceramide and sphingosine, respectively, are involved in the regulation of inflammatory response (61). Because ceramide, C1P, sphingosine, and S1P are interconvertible, we also determined their levels to distinguish whether the observed effects are from ceramide itself, or due to its conversion to its bioactive metabolites. Results showed that there is no age difference in adipocyte sphingosine (Fig. 9A) and S1P levels (Fig. 9B), whereas old adipocytes had a lower level of C1P compared with that of young (Fig. 9B). Thus, in the subsequent experiments we focused on ceramide. To further investigate whether altered cellular levels of ceramide play a role in the age-associated up-regulation of IL-6, we used currently available approaches to manipulate cellular ceramide. We found that increasing ceramide levels in adipocytes of both young and old mice by the addition of

FIGURE 9. Old adipocytes have higher levels of ceramide and sphingomyelin compared with those from young mice. Epididymal adipose tissues were collected from young and old mice and adipocytes were isolated using collagenase digestion. Adipocytes were incubated for 24 h to become quiescent and then collected into glass extraction tubes. Sphingolipids were analyzed as described in the Materials and Methods. Sphingolipid content was normalized with total cellular DNA. Results represent mean ± SE, n = 2 for young and n = 3 for old mice with triplicates for each. SM, Sphingomyelin; Cer, ceramide; GlcCer, glucosylceramide; SO, sphingosine.

FIGURE 10. Ceramide is involved in increased IL-6 and TNF-α production by adipocytes of young and old mice. Epididymal adipose tissues were collected from young and old mice and adipocytes were isolated using collagenase digestion. Adipocytes were incubated for 24 h to become quiescent before being incubated with C2 or C6 ceramide (15 μM) or SMase (0.1 unit/ml) for another 24 h. Culture medium was collected and analyzed for IL-6 and TNF-α using ELISA. TNF-α was not detectable. Production of IL-6 was normalized with total cellular DNA (A). To inhibit ceramide production, quiescent adipocytes were incubated with 50 nM myriocin (inhibitor of de novo ceramide synthesis), 50 μM Me-SM (inhibitor of neutral SMase), or both for 1 h and then LPS (0.2 μg/ml) was added to stimulate cells for 24 h. Culture medium was collected and analyzed for IL-6 and TNF-α using ELISA. Production of IL-6 and TNF-α was normalized with total cellular DNA. Results represent mean ± SE, n = 4. Significant age differences were detected in every treatment at p < 0.05 by Student’s t test. Different letters (lower and upper cases for young and old, respectively) denote significant difference due to treatment at p < 0.05 by Student’s paired t test with Bonferroni adjustment.
and TNF-α (19, 20). Thus we hypothesized that ceramide may increase IL-6 through NF-kB. Bay 11-7082 inhibits NF-kB (2 or 5 μM) for 30 min and then further incubated in the presence and absence of SMase (0.1 unit/ml) or LPS (0.2 μg/ml) for 24 h. Culture medium was collected and analyzed for IL-6 and TNF-α using ELISA. Production of IL-6 and TNF-α was normalized with total cellular DNA. A and B show SMase- and LPS-induced cytokine production, respectively. TNF-α was not detectable in unstimulated and SMase-treated cells. Results represent mean ± SE, n = 8–10. Significant age differences were observed in every treatment at p < 0.05 by Student’s t test. Different letters (lower and upper cases for young and old mice, respectively) denote significant difference due to treatment at p < 0.05 by Student’s paired t test with Bonferroni adjustment. *, Significantly different from LPS alone at p < 0.05 or smaller by Student’s t test. N.D., Not detectable.

Unstimulated IL-6 production was also reduced by Bay 11-7082 (data not shown). TNF-α was not detectable in unstimulated and SMase-treated cultures. LPS-stimulated TNF-α production was greatly reduced in old adipocytes while it became undetectable in young adipocytes (Fig. 11B). These results suggest that ceramide plays an important role in the age-associated up-regulation of IL-6 in adipocytes and that this effect of ceramide may be mediated through the enhancement of NF-kB activation.

**Discussion**

Fat, muscle, and liver are three major insulin target tissues that control glucose homeostasis. The importance of adipose tissue in controlling insulin sensitivity is demonstrated not only within the adipose tissue but also by its ability to affect insulin sensitivity in muscle and liver (64). Adipocytes, in addition to serving to store lipids, also function as an endocrine tissue by secreting adipokines, a number of inflammatory molecules believed to cause metabolic complications including insulin resistance. Because the incidence of T2D increases with age, we hypothesized that secretion of these inflammatory molecules by adipose tissue may be up-regulated with aging. We show here, for the first time, that the expression of all the inflammation markers tested, namely the classic inflammatory cytokines IL-1β, IL-6 and TNF-α and a hallmark lipid inflammatory mediator, COX-2, are up-regulated in adipocytes with aging. Furthermore, PPAR-γ, a nuclear receptor with anti-inflammatory property, is down-regulated with aging.

Obesity has been shown to be associated with an elevated level of inflammatory molecules. In studies using rodents (particularly rats) as a model to study the effect of aging, body weight and fat...
volume could be a potential confounding factor because old rodents typically have much larger body weight and fat mass than young rodents. To avoid the interference of this factor, young mice used in this study were at the age of 5 to 6 mo so that their body weight would be closer to that of old mice, and very heavy old mice were not included. As shown in Table I, although the old mice have statistically higher body weight and fat volume compared with young mice, the actual differences were rather small (18% difference in body weight) by rodent standards and these old mice are not considered obese. In addition, we did not observe any correlation between body weight or fat mass and COX-2, IL-1β, IL-6, and TNF-α (r = 0.39, 0.44, 0.34, and 0.35, respectively, all at p = 1.0 for body weight, and r = 0.57, 0.59, 0.65, and 0.59, respectively, at p = 0.44 to 1.0 for fat mass) in these mice. Moreover, in a separate study (D. Wu, Z. Ren, W. Guo, M. Pae, S. N. Meydani, manuscript in preparation), although young (6 mo; n = 13) and old (24 mo; n = 11) mice had similar body weights (43.0 ± 0.86 and 41.7 ± 2.35 g, respectively) after being fed a control diet for obesity research (10% kilocalories from fat; Research Diets) for 20 wk, the old mice exhibited higher expression of mRNA for the above-mentioned inflammatory molecules in adipose tissue as well as IL-6 and TNF-α production by isolated adipocytes (data not shown). Thus, these data indicate that the small percentage difference in body weight and fat mass between young and old mice observed in the current study is not the main contributor to the higher level of inflammatory products in the old mice. Rather, these results strongly suggest that aging per se has an independent impact on the development of an inflammatory state in adipose tissue. More supportive evidence for the suggestion that there are varied mechanisms for obesity- and age-induced adipose inflammation is that there is no significant difference in the sizes of adipocytes (Fig. 2) between young and old mice in contrast to the observation that adipocytes from obese animals are larger than those in lean animals.

Mφ are perhaps the most significant source for the majority of inflammatory mediators in the body. Increased Mφ infiltration in adipose tissue is shown to be correlated with adiposity and may be the major cellular source of inflammatory changes (11, 12) in obesity. Similar to the finding reported in obesity, we found that aging causes increased expression of inflammatory mediators (IL-1β, IL-6, TNF-α, and COX-2). However, unlike the observation in obesity, we did not find increased Mφ infiltration with aging as demonstrated by the immunohistochemistry of adipose tissue and the flow cytometry of SVC stained for Mφ (F4/80^+^ cells). This is compatible with the lack of difference in MCP-1 production by young and old adipocytes in this study. Our observation that old mice had higher yield of SVC from the same amount of fat tissue compared with young mice is in accordance with the previous finding that aging impairs adipocyte maturation, resulting in the presence of more preadipocytes in the fat tissue of old rats compared with that of young (6). Furthermore, the observation that adipose tissue from old mice have lower mRNA levels of PPAR-γ, a nuclear receptor shown to promote adipocyte differentiation, is also in agreement with the results reported by Karagiannides et al. (6).

To determine the cellular origin of the up-regulated inflammation state with age, we showed that adipocytes from old mice produced significantly more IL-6, TNF-α, and PGE_2_ than those from young mice, whereas the production of these inflammatory mediators in SVC was not different between young and old mice. SVC are a complex of different cells including at least fibroblasts, preadipocytes, Mφ, endothelial cells, and epithelial cells among other cell types. Although these results indicate that SVC collectively do not exhibit age-related increase in inflammatory products, this does not rule out the possibility that individual components might exhibit age-related differences. Because Mφ have been indicated to be the main contributors to obesity-induced adipose tissue inflammation, we determined intracellular cytokine levels in Mφ in a SVC fraction by FACS. We demonstrated that Mφ in the adipose tissue of old mice produced more IL-6 and TNF-α compared with those in young mice. This finding, combined with a lower percentage of Mφ in SVC isolated form old compared with young mice, might partly explain the lack of age-related differences in the production of inflammatory molecules by the same number of SVC. However, adipose tissue in old mice contain significantly more SVC compared with young; thus, SVC as a whole may still contribute to up-regulated inflammation in adipose tissue with aging.

TNF-α is the first proinflammatory cytokine that has been shown to be markedly increased in adipocytes of obese animals, and its neutralization by a soluble TNF-α receptor leads to improved insulin sensitivity in these animals (53). In some studies, however, no detectable level or increase of circulating TNF-α was found in obese subjects (57) although their adipose tissue had higher TNF-α mRNA or protein levels (57, 65–67), suggesting that TNF-α may be a powerful autocrine and paracrine regulator. By contrast, IL-6 appears to be released systemically by adipose tissue, which contributes up to one-third of circulating IL-6 (68). IL-6 exhibits the strongest correlation with insulin resistance and T2D (30, 67, 69). IL-6 induces insulin resistance in hepatocytes (70), skeletal muscle (52), and adipocytes (51), the three major target tissues for insulin function. Therefore, elevated levels of TNF-α and IL-6 in adipose tissue of old mice might be an important contributor to the age-associated insulin resistance and higher incidence of T2D.

All of the inflammatory mediators tested in this study showed an increased expression with aging, suggesting that a common mechanism may be involved. The transcription of genes for all of these inflammatory mediators is regulated by NF-κB, whereas PPAR-γ antagonizes NF-κB-associated activation. To date, no information is available regarding the age-related change in adipocyte NF-κB and PPAR-γ activity. However, an age-associated up-regulation in NF-κB binding activity has been shown to be present in various types of mouse tissues, and this elevated NF-κB activity was diminished by a PPAR agonist (40, 71). Thus, it is likely that activation of NF-κB in adipocytes is up-regulated with aging, resulting in increased expression of its target genes and, consequently, secretion of their products. Of interest is the observation that IkB kinase-β/NF-κB signaling pathway is indicated as a potential mediator in the cross-talk between inflammation and insulin resistance (31, 48, 72). Our results support the involvement of NF-κB in the age-related increase in IL-6 and TNF-α production by adipocytes, because blocking NF-κB activation significantly decreased IL-6 and TNF-α production in adipocytes.

Increasing evidence has revealed that ceramide may be a key factor in mediating obesity-induced insulin resistance via both metabolic (i.e., free fatty acids) and inflammatory (i.e., cytokines) pathways (21). We hypothesized that ceramide is involved in the age-associated inflammatory state in adipose tissue. Our findings in the current study support this hypothesis. First, old adipocytes contain more ceramide and its precursor sphingomyelin compared with young adipocytes. Second, IL-6 production was increased by elevating ceramide levels through the addition of synthetic short-chain ceramides or SMase that hydrolyze sphingomyelin to give rise to endogenous ceramide. Third, inhibition of either de novo ceramide synthesis using the SPT inhibitor myriocin or of nSMase using Me-SM significantly reduced IL-6 and TNF-α production. When both inhibitors were added there was an additive effect, suggesting that ceramide generated from both de novo synthesis and sphingomyelin hydrolysis contributes to the stimulation of IL-6 and TNF-α production. Finally, blocking NF-κB activation
reduced LPS-stimulated IL-6 and TNF-α production and SMase-induced IL-6 production. Increased tissue ceramide content with aging has been reported (32–35), but the mechanism underlying this phenomenon remains elusive. Cellular ceramide can be generated through the hydrolysis of sphingomyelin catalyzed by SMase or its de novo synthesis under action of several enzymes, including the rate-limiting SPT, from palmitoyl CoA and serine. Both SMase and SPT seem to be regulated by the redox state.

nSMase activity can be activated by decreasing total glutathione (GSH) and the GSH/glutathione disulfide (GSSG) ratio and increasing hydrogen peroxide, and it can be inhibited by increasing GSH (73–77). Aging is associated with increased oxidative stress and, in particular, the age-related loss of GSH and a reduced GSH/GSSG ratio have been shown in different tissues (78–81). Age-related elevation in nSMase activity and ceramide level are attributed to declined GSH content in the endothelium of rats (35); increased oxidative stress is associated with higher ceramide accumulation in the brains of old mice, which can be blocked by pretreatment with a SPT inhibitor or the antioxidant cumulation in the brains of old mice, which can be blocked by pretreatment with a SPT inhibitor or the antioxidant cAMP, respectively.

In this study, we found an age-associated elevation in both TNF-α and IL-6 production when ceramide synthesis or NF-κB activation was blocked. Together with previous reports stating that ceramide induces NF-κB activation (17, 19, 84) and the fact that the transcription of TNF-α and IL-6 is controlled by NF-κB, we propose that the age-associated up-regulation of both ceramide and NF-κB work sequentially to promote the production of inflammatory molecules. These inflammatory molecules, in turn, contribute to the induction of insulin resistance, a key factor in the etiology of T2D.

In summary, we report, for the first time, that an inflammatory state is developed in adipose tissue with aging as evidenced by increased expression of classic inflammatory mediators and decreased expression of the anti-inflammatory nuclear receptor PPAR-γ. Unlike the obesity-induced inflammatory state in which Mφ are the main source of the inflammatory changes, adipocytes are the major contributor to the age-related increase in adipose tissue inflammatory products. Our data further suggest that ceramide and NF-κB may underlie the observed inflammatory state. In addition, adipocyte-produced signals up-regulate inflammatory cytokine production by Mφ, increasing the impact of adipocytes on age-related inflammation. These findings are important in understanding the basis of age-related inflammation and its cellular components and could provide a clue for searching the underlying mechanisms of the age-related increase in insulin resistance and T2D incidence. Further studies are needed to establish a direct link between age-associated inflammation and insulin resistance.

Acknowledgment
We thank Dr. Kate Claycombe (Michigan State University) for advice on adipose tissue extraction and adipocyte isolation.

Disclosures
The authors have no financial conflict of interest.

References


