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

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

References
This article cites 86 articles, 39 of which you can access for free at: http://www.jimmunol.org/content/179/7/4829.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Aging Up-Regulates Expression of Inflammatory Mediators in Mouse Adipose Tissue

Dayong Wu,2* 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 adipose tissue-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.

1 Abbreviations used in this paper: T2D, type 2 diabetes; ACM, adipocyte-conditioned medium; CIP, ceramide-1-phosphate; COX, cyclooxygenase; GSH, glutathione; GSSG, glutathione disulfide; iNOS, inducible NO synthase; LDH, lactate dehydrogenase; MeSM, 3-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; nSMase, neutral sphingomyelinase; PPAR, peroxisome proliferator-activated receptor; RIA, radioimmunoassay; S1P, sphingosin-1-phosphate; SPT, serine palmitoyltransferase; SVC, stromal vascular cell.

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

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institute of Aging Grant R01-AG009140-10A1, Office of Dietary Supplement, the U.S. Department of Agriculture, Agriculture Research Service under contract number 53-K06-01 (to S.N.M.), and the Obesity Research Center on Aging, Tufts University, 711 Washington Street, Boston, MA 02111. *National Institute of Communicable Disease Control and Prevention, China Center for Disease Control, Beijing, China; and †School of Biology and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332.

2 Address correspondence and reprint requests to Dr. Dayong Wu, Nutritional Immunology Laboratory, Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, 711 Washington Street, Boston, MA 02111. E-mail address: dayong.wu@tufts.edu

3 Abbreviations used in this paper: T2D, type 2 diabetes; ACM, adipocyte-conditioned medium; CIP, ceramide-1-phosphate; COX, cyclooxygenase; GSH, glutathione; GSSG, glutathione disulfide; iNOS, inducible NO synthase; LDH, lactate dehydrogenase; MeSM, 3-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; nSMase, neutral sphingomyelinase; PPAR, peroxisome proliferator-activated receptor; RIA, radioimmunoassay; S1P, sphingosin-1-phosphate; SPT, serine palmitoyltransferase; SVC, stromal vascular cell.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
tissue. It is thus speculated that the low-grade inflammatory signal delivered by adipocytes in the obese may act on Mφ to propagate the initial inflammatory signal (14, 15).

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, cytochrome oxidase (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 overexpression 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/6 NIA 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 librum. 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. The tissues were immediately put in liquid nitrogen and then stored at −80°C before RNA extraction. Adipose tissue was homogenized using a A3500 kit (Promega) reverse transcription system. Tissue samples (80 mg) were dissolved in 1 N NaOH at 80°C before RNA extraction. Adipose tissue was homogenized using a A3500 kit (Promega) reverse transcription system. Tissue samples (80 mg) were dissolved in 1 N NaOH at 80°C. ACM isolation and incubation with adipocyte-conditioned medium (ACM)

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 using a Quantos cell proliferation assay kit from Stratagene and total pro-

\[
\text{IL-6 and TNF-}\alpha \text{ inhibitors myriocin (50 nM) sphingomyelinases (nSMase; 0.1 U/ml) (Sigma-Aldrich) were added to }
\]
using radioimmunoassay (RIA) as previously described (42). To normalize

be measured by the amount of 490 nm absorbance, which is directly pro-

assay (CellTiter 96 Aqueous One Solution cell proliferation assay kit; 

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium (MTS)

2-(4-sulfopheny)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution cell proliferation assay kit; Promega). In this assay, MTS tetrazolium is converted to a formazan product by metabolically active cells and the quantity of this product can be measured by the amount of 490 nm absorbance, which is directly propor-

tional to the number of living cells. The cell viability was further con-

firmed with lactate dehydrogenase (LDH) release assay using the CytoTox 96 nonradioactive cytotoxicity assay (Promega). In this assay, LDH release into culture medium and present in the cells was measured to cal-

culate the percentage of LDH release [medium LDH/(medium LDH + cellular LDH) \times 100]. Cellular LDH was determined after cells were lysed using lysis solution. The total number of cells was normalized with total cellular DNA.

**Intracellular IL-6 and TNF-α in adipose tissue Mφ**

SVC isolated as described above were stimulated with LPS (0.2 µg/ml) for 24 h. Four hours before collection, brefeldin A (10 µg/ml; Sigma-Aldrich) was added to block cytokine transport out of the cells. Cells were 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% para-

formaldehyde 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 ana-

lyzed by a FACSCalibur flow cytometer (BD Biosciences).

**Sphingolipid analysis**

The methods for sphingolipid analysis have been described in depth pre-

viously (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 com-

plex lipids the extract was acidified with 3 µl of glacial acetic acid and the liquids 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 sphingolip-

idns in adipocytes were normalized 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 in-

crease in inflammatory molecules, we first determined the mRNA expres-

sion of inflammatory molecules compared with that of young mice. 

Expression of inflammatory molecules in different fat depots and found that

expression of these molecules in different fat depots and found that

those from young lean mice (Fig. 1). We also sampled mRNA

expression of IL-1β, IL-6, TNF-α, and COX-2 lower mRNA

expression of PPAR-γ, a nuclear receptor possessing anti-

inflammatory and insulin-sensing 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., peripheral fat, as well as s.c. fat (inguinal adipose tissue) from old mice, also had a higher expres-

sion 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.** Epididymal adipose tissue of old mice has higher mRNA expression of proinflammatory mediators compared with that of young mice. Epididymal adipose tissues were collected from young and old mice. Total RNA in adipose tissues 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, \( n = 6–9 \) in young mice and \( n = 7–10 \) in old mice. AT, Adipose tissue.

**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 (x10^4 cells)</th>
<th>SVC/SVC (%)</th>
<th>Total Mφ (x10^4 cells/g)</th>
<th>SVC/AT (x10^4 cells/g)</th>
<th>Mδ/AT (x10^4 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 of 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

![FIGURE 2. Histological comparison of adipose tissue between young and old mice. Formalin-fixed and paraffin-embedded epididymal adipose tissues from young and old mice were stained with rat anti-mouse F4/80 Ab and counterstained with Mayer’s hematoxylin. The photomicrographs are representative sections of young and old mice.](http://www.jimmunol.org/)

![FIGURE 3. MCP-1 production by adipocytes is not different between 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 stimulated with LPS (0.2 μg/ml) for another 24 h. Culture medium was collected and analyzed for MCP-1 using ELISA, and the concentration of MCP-1 was normalized with total cellular DNA. Results represent mean ± SE, n = 10.](http://www.jimmunol.org/)
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. ACM was 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
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](image)

**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](image)

**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.
nSMase, which elevates endogenous ceramide levels, or by the addition of exogenous short-chain C2 and C6 ceramides significantly increased IL-6 production (Fig. 10A). However, TNF-\(\alpha\) was not detectable under these conditions. The addition of nSMase increased IL-6 production in young adipocytes to a level similar to that seen in untreated old adipocytes. In accordance with this, reducing ceramide levels by adding either myriocin, an inhibitor of SPT and thus de novo ceramide synthesis, or Me-SM, a specific inhibitor of nSMase that converts sphingomyelin to ceramide, or a combination of both significantly decreased IL-6 (Fig. 10B) and TNF-\(\alpha\) (Fig. 10C) production in young and old adipocytes stimulated by LPS. The addition of Me-SM and myriocin to old adipocytes reduced IL-6 and TNF-\(\alpha\) production to a level closer to that seen in young adipocytes. These data suggest that both de novo synthesis of ceramide and its hydrolytic release from sphingomyelin are involved in increased IL-6 and TNF-\(\alpha\) production by adipocytes.

Ceramide-induced IL-6 and TNF-\(\alpha\) production is mediated through NF-\(\kappa\)B

IL-6 and TNF-\(\alpha\) are transcriptionally controlled by NF-\(\kappa\)B (62), and ceramide is shown to both directly enhance NF-\(\kappa\)B activation (17–19) and potentiate other stimulant-induced NF-\(\kappa\)B activations (19, 20). Thus we hypothesized that ceramide may increase IL-6 and TNF-\(\alpha\) production through the NF-\(\kappa\)B pathway. In this study we used the inhibitor Bay 11–7082 to block NF-\(\kappa\)B activation. Bay 11–7082 inhibits NF-\(\kappa\)B activation by blocking IkB phosphorylation (63), and its specificity and efficacy was verified in our previous study (19). Both LPS- and SMase-induced IL-6 production was diminished in the presence of Bay 11-7082 (Fig. 11). Unstimulated IL-6 production was also reduced by Bay 11-7082 (data not shown). TNF-\(\alpha\) was not detectable in unstimulated and SMase-treated cultures. LPS-stimulated TNF-\(\alpha\) 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-\(\kappa\)B 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-1\(\beta\), IL-6 and TNF-\(\alpha\) and a hallmark lipid inflammatory mediator, COX-2, are up-regulated in adipocytes with aging. Furthermore, PPAR-\(\gamma\), 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 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 inflammatory state in adipose tissue. More supportive evidence for the 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 Karagianides 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 PGE₂ 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, Mₐφ, 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 Mₐφ have been indicated to be the main contributors to obesity-induced adipose tissue inflammation, we determined intracellular cytokine levels in Mₐφ in a SVC fraction by FACS. We demonstrated that Mₐφ 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 Mₐφ 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 IκB 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...