Translational Article

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Fatty Acid-Induced Production of Plasminogen Activator Inhibitor-1 by Adipose Macrophages Is Greater in Middle-Aged Versus Younger Adult Participants

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Background. Human aging is associated with heightened risk of diabetes and cardiovascular disease. Increased fat mass may contribute to age-related diseases by harboring inflammatory macrophages that produce metabolically important proteins such as plasminogen activator inhibitor-1 (PAI-1). Elevated PAI-1 concentrations have been implicated in the pathogenesis of such aging-related conditions as insulin resistance, obesity, and atherosclerosis. We have previously reported that increased plasma free fatty acid (FFA) concentrations augment both circulating PAI-1 concentrations and PAI-1 production by adipose tissue macrophages (ATMs).

Methods. Because increasing age is associated with increased infiltration and reactivity of adipose macrophages, we performed euglycemic-hyperinsulinemic clamp studies and adipose tissue biopsies with and without elevated FFA concentrations in 31 nondiabetic participants stratified by age, to determine whether middle-aged individuals manifest heightened insulin resistance and PAI-1 production by ATMs in response to elevated nutrient signals relative to their young adult peers.

Results. We observed that elevating FFA concentrations under euglycemic-hyperinsulinemic clamp conditions induced the same degree of insulin resistance in both middle-aged and younger body mass index-matched adults, whereas systemic PAI-1 concentrations were significantly increased in the middle-aged group. Likewise, elevated FFA and insulin concentrations induced larger increases in PAI-1 gene expression in the whole fat and ATMs of middle-aged compared with younger adult participants.

Conclusions. These studies reveal a heightened adipose inflammatory response to increased FFA and insulin availability in middle-aged individuals relative to younger adults, suggesting that increased susceptibility to the effects of fatty acid excess may contribute to the pathogenesis of age-related diseases.

Key Words: Insulin resistance—Plasminogen activator inhibitor-1—Free fatty acids.

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Type 2 diabetes mellitus (T2DM) and prediabetes have a combined prevalence of >75% among adults aged 65 and older in the United States (1). The “metabolic syndrome of aging” defines a cluster of abnormalities including insulin resistance, visceral obesity, and increased risk of cardiovascular and other age-related diseases (2–6). Fat-derived cytokines and acute phase reactants have been implicated in the pathogenesis of these diseases (7–10), and advancing age is associated with both increased fat mass and elevated circulating “adipokine” levels, such as plasminogen activator inhibitor-1 (PAI-1) (11–14).

PAI-1 is a serine protease inhibitor that facilitates blood clotting (15). PAI-1 concentrations are correlated with increased cardiovascular disease risk (16,17) and may contribute to
the development of insulin resistance and obesity (18–20). Adipose tissue macrophages (ATMs) express high levels of PAI-1, suggesting that macrophage infiltration into fat contributes importantly to adipose PAI-1 production (21). Increasing age is associated with elevated circulating FFA concentrations (22), which rapidly increase the expression and production of PAI-1 in fat (23). Moreover, aging heightens FFA-induced insulin resistance and activation of ATMs in rats (24).

Although the age at which these risks increase has not been firmly established, there is some suggestion that these changes may already be present by middle age. Therefore, we hypothesized that the elevated FFA concentrations found in middle-aged versus younger adults can lead to macrophage activation and contribute to the pathogenesis of age-related diseases. The current report examines how age determines the impact of elevated FFA on PAI-1 production and insulin action in humans and explores the role of adipose macrophages in this process.

**METHODS**

**Recruitment and Exclusion Criteria**

A total of 31 healthy volunteers were recruited from the Clinical Research Center (CRC) database and by local advertising. All participants with a history of diabetes mellitus, impaired glucose tolerance, hypertension, heart disease, cerebrovascular disease, seizures, bleeding disorders, muscle disease, or smoking were excluded. Women in the childbearing age group were allowed to participate in the study if a pregnancy test within a week of the clamp study was negative. Some data from a subgroup of the younger participants group were presented in a previous publication (21).

Prior to their enrollment in the study, the purpose, nature, risks, and benefits of the study were explained to all participants and their voluntary, informed, written consent was obtained. All participants had a screening visit that included a full history and physical examination, an oral glucose tolerance test (OGTT) and additional laboratory tests, including serum electrolytes, blood urea nitrogen and creatinine, prothrombin time and partial thromboplastin time, liver function tests, and lipid profile, as well as a screening urinalysis and blood pressure measurements. The OGTT consisted of a 75-g oral glucose challenge with fasting and 2-hour venous blood samples for plasma glucose measurements. Participants in the young and middle-aged group were matched for body mass index (BMI) and fat free mass (Table 1). All studies and procedures were performed under protocols reviewed and approved by the Institutional Review Board of Albert Einstein College of Medicine.

**In Vivo Clamp Studies**

Each participant was instructed to follow their usual diet plan and not to change their level of activity while participating in the studies. Each participant was studied on two separate occasions (at least 1 month apart) under the following conditions:

1. Five-hour saline infusion control study (SAL): 5-hour euglycemic-hyperinsulinemic “clamp” studies, as described later.
2. Five-hour Liposyn infusion study (LIP): Liposyn was infused at 1.5 mL/min for the entire 5 hours of the euglycemic-hyperinsulinemic clamp studies, with the addition of heparin at 0.4 U/kg/min to activate lipoprotein lipase.

Following an overnight fast, all participants were admitted to the CRC on the day of the clamp study, and an intravenous catheter was inserted into the left arm for infusions and the right arm for blood sampling. To obtain arterialized venous blood samples, the right hand was maintained at 65°C in a thermo-regulated plexiglass box or a warming blanket. Insulin infusions (Humulin R, Eli Lilly, Indianapolis, IN) were initiated at $t = 0$ at 80 mU/m²/min for 10 minutes (prime) followed by 40 mU/m²/min for the rest of the study. Plasma glucose was measured every 5–10 minutes by a Beckman Glucose analyzer (Beckman Coulter, Fullerton, CA) and maintained at ~90 mg/dL by variable 20% dextrose infusions. The amount of glucose required to maintain euglycemia or glucose infusion rate (GIR) was calculated per kg of fat free mass (FFM) measured by bio-impedance according to manufacturer’s recommended method (Quantum II, RJL Systems). GIR was used as a measure of insulin resistance, as we have previously shown that GIR correlates well with rates of glucose disappearance measured with tritiated glucose. Samples were collected every 15–60 minutes to measure plasma insulin, total FFA, and PAI-1 concentrations. All infusions were stopped at $t = 300$ minutes. The participants were given a standard meal and plasma glucose levels were monitored for ~60 minutes.

**Adipose Tissue Biopsies**

Adipose tissue biopsies were obtained after 5 hours of LIP or SAL infusion ($t = 240–300$ minutes). A 0.25-cm cutaneous incision was performed under local anesthesia (Lidocaine 1%) and ~5 g of adipose tissue was obtained by aspiration technique (25) from subcutaneous abdominal fat between the umbilicus and iliac crest. The biopsy specimens were immediately homogenized in Trizol (Invitrogen Technologies, Carlsbad, CA) to inhibit any RNAase activity, and subsequently stored at ~80°C.

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*p < .05 young vs middle-aged participants.
The time course of adipose biopsies was selected based upon important in vivo human observations regarding the effect of FFA concentrations on PAI-1 production. Specifically, Krebs and coworkers (26) reported a 2.6-fold elevation in plasma PAI-1 concentrations following 6 hours of elevated FFA/triacylglycerol concentrations. Conversely, lowering FFA concentrations in obese human participants for only 2 hours lowered plasma PAI-1 concentrations by ~42%, and this effect persisted with 4 hours of FFA lowering (27). Thus, the decision to perform adipose biopsies at 2 and 5 hours was based upon these preceding observations, with the objective of determining whether effects of FFA on PAI-1 production from adipose cell types are both acute and persistent.

Adipose Tissue Separation

Subcutaneous adipose tissue samples were immediately washed and digested with collagenase type 1, and adipocytes were separated from the stromal-vascular fraction, as previously described (21). Separated adipocytes and macrophages were washed with PBS, stored in Trizol, and analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Using this method, we found that we recover >80% of the CD68 positive cells from the stromal vascular cell fraction of fat (28). Furthermore, the separation of adipocytes from whole adipose tissue is >90%.

Real-Time RT-PCR

From the samples obtained as described previously, total RNA was isolated with RNasy Lipid Tissue (Qiagen, Valencia, CA). cDNA was synthesized using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen Technologies, Carlsbad, CA). Gene expression was studied by quantitative, real-time PCR using the specific protocol for the LightCycler instrument (Roche Diagnostics). SYBRGreen1 dye (Roche Diagnostics, Indianapolis, IN) was used for fluorescent detection of double-stranded DNA and housekeeping genes GAPDH, RPL19, and 18S were used, as previously described (21).

Plasma Hormone and Substrate Determinations

Plasma insulin was measured by radioimmunoassay and plasma FFA concentrations by an acyl-CoA oxidase-based colorimetric kit (Wako, Osaka, Japan). Lipase inhibitors were not added to the sample collection tubes, and it is possible that this may have resulted in artificially increased FFA concentrations in this study. Our previous study (21) with the same infusion rate of Liposyn showed FFA concentrations of ~800 µM/L. Plasma concentrations of PAI-1 were measured by Enzyme-Linked immunosorbent assay (Trinity Biotech, Dublin, Ireland).

Statistical Analysis

Statistical analysis of the data was performed using PSAW statistical software Version 18.0 (IBM, Somers, NY). Paired Student’s t tests were used for all the comparisons between the Liposyn versus saline control studies and unpaired t tests for comparison between the middle-aged and younger groups. P < .05 was considered significant. All data are expressed as means ± SEM unless otherwise specified.

RESULTS

Hyperinsulinemic Clamp Studies

Nondiabetic participants (n = 31) were studied for their response to elevated FFA concentrations in order to examine responses to this metabolic challenge in younger (13M/8F, age 25 ± 0.8 years, age range 19–32 years, BMI 26.3 ± 1.0 kg/m²) versus middle-aged individuals (9M/1F, age 52.9 ± 2.5 years, age range 44–68 years, BMI 27.9 ± 1.6). Baseline participant characteristics are presented in Table 1. Of note, basal FFA concentrations were higher in the middle-aged participants (605.1 ± 213.9 µM/L in middle-aged participants vs 361.3 ± 35.8 µM/L in younger participants, p = .02). Liposyn and heparin infusions elevated FFA concentrations to ~1200 µM in both the younger adult and middle-aged groups. Because the same infusion rates of Liposyn and heparin raised FFA concentrations to ~800 µM in our previous study (21) achieving concentrations ~2- to 3-fold above normal, it is likely that the higher FFA concentrations observed in this study were an artifact of continuing in vitro lipolysis in the absence of lipase inhibitors. During the 5-hour SAL studies, insulin suppressed FFA concentrations to ~60 µM. In younger participants, the mean GIR decreased significantly from 11.5 ± 0.4 mg/kgFFM/min during the SAL studies to 5.6 ± 0.6 mg/kgFFM/min during the LIP studies (p < .00001). In middle-aged participants, the mean GIR decreased from 9.5 ± 0.7 mg/kgFFM/min in the SAL studies to 5.9 ± 0.6 mg/kgFFM/min in the LIP studies (p = .008). Of note, these hyperinsulinemic clamp studies were designed to study peripheral (nonhepatic) insulin sensitivity, as endogenous glucose production would be expected to be suppressed at these high insulin infusion rates. Although the middle-aged group tended to have a lower GIR at baseline compared with the younger group, this difference did not reach statistical significance (p = .09) (Figure 1A).

Analysis of plasma insulin levels during the second hour and the final hour of the clamp studies revealed that insulin levels did not differ between saline and Liposyn studies (at 120 minute, SAL = 91.9 ± 7.9 vs LIP = 98.2 ± 10.2 and in the last hour SAL = 98.2 ± 9.7 vs LIP = 90.1 ± 7.6 µU/mL, p = NS). As per the study design, plasma FFA concentrations were higher at LIP studies compared with SAL (at 120 minute, SAL = 79.4 ± 2.7 vs LIP = 930.7 ± 24.5 and in the last hour SAL = 57.8 ± 2.4 vs LIP = 1251 ± 40.1 µM/L, p < .05). During the SAL studies, insulin suppressed circulating FFA (57.3 ± 20.1 µM/L in younger participants and 69.6 ± 24.6 µM/L in middle-aged participants, p = NS) and reduced plasma PAI-1 to similar concentrations in both groups of participants (14.2 ± 2.3 ng/mL in the younger group vs 17.7 ± 1.8 ng/mL in the middle-aged group, p = NS,
Elevating FFA concentrations in the presence of insulin in the LIP studies raised plasma PAI-1 to substantially higher concentrations in the middle-aged participants (25.8 ± 5.5 ng/mL in the younger group vs 45.6 ± 6.3 ng/mL in the middle-aged group; *p < .05, Figure 1B). This suggested a greater susceptibility to enhanced PAI-1 production in the middle-aged participants when they were exposed to increased fatty acid flux.

**Adipose Tissue Biopsies**

In a subset of BMI-matched younger and middle-aged participants (younger participants: *n* = 8, age 24 ± 1 years, age range 19–31 years, BMI = 28 ± 2 kg/m²; middle-aged participants: *n* = 9, age 57 ± 3 years, age range 45–68 years, BMI = 32 ± 2 kg/m²), subcutaneous abdominal fat was biopsied and PAI-1 gene expression was analyzed by RT-PCR at 2 hours of Liposyn infusion. Identical fat biopsies followed by analysis of PAI-1 gene expression by RT-PCR were performed at 5 hours of Liposyn infusion in a separate subset of BMI-matched younger and middle-aged participants (younger participants: *n* = 7, age 23 ± 2 years, age range 19–31 years, BMI = 30 ± 1 kg/m²; middle-aged participants: *n* = 8, age 54 ± 3 years, age range 42–68 years, BMI = 28 ± 2 kg/m²). PAI-1 gene expression in whole fat

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**Figure 1.** (A) Age-dependent effects of Liposyn or saline infusion on glucose infusion rate (GIR, a measure of insulin sensitivity): GIR was measured during euglycemic-hyperinsulinemic clamp studies in groups of younger (*n* = 21) and middle-aged (*n* = 10) participants. Elevating FFA concentrations in both groups significantly reduced GIR, indicating development of insulin resistance (**p < .001 for both groups**). At baseline, middle-aged participants were somewhat insulin resistant (*p = .09 middle-aged vs young*). Insulin resistance appears to reach a “maximum” with FFA infusion as this led to both groups requiring comparable glucose infusion rates.

(B) Age-dependent effects of Liposyn or saline infusion on plasma PAI-1 concentrations: PAI-1 concentrations did not differ substantially in younger participants (*n* = 21) following Liposyn infusion vs saline infusion. However, PAI-1 concentrations were significantly higher in middle-aged participants (*n* = 10) with Liposyn infusion than with saline infusion (*p < .05*).
did not increase with elevated FFA and insulin concentrations in the younger participants at both 2 and 5 hours (1.1 ± 0.08 and 0.73 ± 0.12 fold, respectively) but was significantly elevated in the middle-aged participants (2 hours = 2.8 ± 0.6 fold and 5 hours = 1.6 ± 0.1 fold, \( p = .035 \) and \( .0002 \), respectively, for 2 and 5 hours vs younger group) (Figure 2A and B). This is noteworthy, because we previously reported the ability of FFA to increase PAI-1 production by ATMs in a heterogeneous population (21).

Therefore, we hypothesized that the macrophages of middle-aged individuals would manifest a heightened responsiveness to FFA. Although Liposyn infusion increased PAI-1 expression 1.9-fold at 2 h in macrophages from the middle-aged participants \( (n = 7) \) (Figure 3), the response was blunted in macrophages from the younger participants \( (n = 8) \) (0.7-fold, \( p < .001 \)) suggesting a heightened response to free fatty acids (FFA) in middle-aged versus younger adults. At 5 hours, the differential response was
Discussion

This study demonstrated heightened susceptibility to key metabolic effects of fatty acids in middle-aged versus younger adult humans. We used euglycemic-hyperinsulinemic clamp studies to compare insulin sensitivity and adipose tissue inflammation in young versus middle-aged participants in the presence and absence of increased plasma FFA. We demonstrated that basal FFA levels were elevated in the middle-aged participants compared with young participants, with a trend toward reduced insulin sensitivity. Elevated FFA induced the same degree of insulin resistance in both the young and middle-aged participants. However, at comparable FFA and insulin levels, gene expression of PAI-1 was significantly higher in whole adipose tissue and adipose macrophages of middle-aged participants compared with young participants, suggesting a heightened response to the same increase in fatty acid flux. We speculate that this response could play an important role in the development of age-related diseases such as type 2 diabetes and cardiovascular disease.

FFA concentrations have been shown to be elevated in older animals and humans (22,29), as confirmed in the current studies. Increasing age is associated with an impaired ability of fat depots to store fatty acids as triglycerides and form new fat cells (30). Because increasing age is associated with increased visceral fat (31), the resistance of visceral fat to antilipolytic factors such as insulin may be an important contributor to the higher basal FFA concentrations observed in our study (32). Such elevations in FFA concentrations have been shown to cause insulin resistance in rodent studies, due to a variety of mechanisms (33,34). In a seminal study in healthy human participants, elevating FFA concentrations by infusing lipid emulsion was associated with the development of insulin resistance (29), with ~50% reduction in GIR following 5 hours of hyperinsulinemic clamp conditions. In our study, elevated FFA concentrations induced comparable insulin resistance in both young and middle-aged participants.

We also demonstrated that elevated circulating FFAs increase plasma PAI-1 concentrations and significantly induce PAI-1 gene expression in the whole fat of middle-aged participants relative to young adult participants. The effects appeared more pronounced at 2 hours than at 5 hours, suggesting an early induction of gene transcription. Because gene expression of PAI-1 was examined during hyperinsulinemic conditions, acute effects of insulin infusion may have blunted the stimulatory effects of elevated FFA on PAI-1 (21). Separating whole fat into its components demonstrated that some of the upregulation of PAI-1 gene expression seen in whole fat could be attributable to production by ATMs in middle-aged participants, whose expression of PAI-1 increased 1.5- to 2-fold following FFA infusion. Intriguingly, PAI-1 expression in whole fat increased by a greater magnitude relative to the ATM response to Liposyn, suggesting that additional cell types could be responsible for the observed difference in whole

Figure 3. Age-dependent effects of Liposyn on PAI-1 gene expression in adipose macrophages: PAI-1 gene expression was significantly induced in middle-aged participants (n = 7) following Liposyn infusion expressed as fold change relative to saline infusion (*p < .01). PAI-1 expression following Liposyn infusion in younger participants (n = 8) did not show a significant change relative to saline.

not statistically significant (1.5-fold in middle-aged vs 0.9-fold in younger group).
Fat gene expression. Indeed, endothelial cells express substantial amounts of PAI-1 and could have contributed to the response observed in whole fat (23). Moreover, if it had been possible to raise FFA concentrations using a mixture containing more saturated fatty acids, it is likely that the PAI-1 response might have been more exaggerated (26,27).

Thus, the current results demonstrate that the inflammatory response of ATMs to increased plasma FFA concentrations is heightened in middle-aged adults relative to younger adults. It is striking that with the same rises in insulin and FFA, the middle-aged participants demonstrated a marked increase in PAI-1 levels. This indicates that as one transitions from young adulthood to middle age, sensitivity to the undesirable effects of excess FFA and insulin increases. It would be particularly interesting to examine the correlation of PAI-1 mRNA levels with GIR and FFA concentrations. Although our study design with limited data points does not allow for such an analysis, this could be the focus of future studies.

In addition to the relatively small number of participants, this study has other limitations to its generalizability to the metabolic effects of aging. Of note, this study reveals altered inflammatory responses during the transition from young adulthood to middle age, but does not examine whether elderly individuals demonstrate similar or even more exaggerated responses. Further study is therefore needed to determine whether the results of this study would follow the same trajectory in a more elderly population. Additionally, the combination of high FFA and high insulin concentrations during the clamp studies with lipid infusion would generally be considered nonphysiologic, as these two conditions would only occur simultaneously in more extreme insulin resistance. Although fasting FFA concentrations are higher in middle-aged people as well as in obesity, this is in the presence of basal insulin concentrations. Similarly, although clamp-level insulin concentrations may be observed postprandially, this causes FFA levels to fall substantially after a meal (35). It is important to note that our study was primarily designed to examine maximal responses to FFA elevation, rather than to mimic a given physiologic situation.

In conclusion, these studies highlight age-related susceptibility to the effects of fatty acids on the production of PAI-1 by adipose tissue and by adipose macrophages. The current results show that these features can appear as early as middle age and may contribute to the age-related increased frequency of cardiovascular disease and T2DM. These findings have important implications not only for the heightened risk of these diseases as people age, but also demonstrate how the activation of ATMs might contribute to the systemic consequences of fatty acid excess in this population.

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**References**


