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Original Research

Inflammation in Response to n3 Fatty Acids in a Porcine Obesity Model

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Fatty acids have distinct cellular effects related to inflammation and insulin sensitivity. Dietary saturated fat activates toll-like receptor 4, which in turn can lead to chronic inflammation, insulin resistance, and adipose tissue macrophage infiltration. Conversely, n3 fatty acids are generally antiinflammatory and promote insulin sensitivity, in part via peroxisome proliferator-activated receptor γ. Ossabaw swine are a useful biomedical model of obesity. We fed Ossabaw pigs either a low-fat control diet or a diet containing high-fat palm oil with or without additional n3 fatty acids for 30 wk to investigate the effect of saturated fats and n3 fatty acids on obesity-linked inflammatory markers. The diet did not influence the inflammatory markers C-reactive protein, TNFα, IL6, or IL12. In addition, n3 fatty acids attenuated the increase in inflammatory adipose tissue $CD16^{-}CD14^{+}$ macrophages induced by high palm oil. High-fat diets with and without n3 fatty acids both induced hyperglycemia without hyperinsulinemia. The high-fat only group but not the high-fat group with n3 fatty acids showed reduced insulin sensitivity in response to insulin challenge. This effect was not mediated by decreased phosphorylation of protein kinase B. Therefore, in obese Ossabaw swine, n3 fatty acids partially attenuate insulin resistance but only marginally change inflammatory status and macrophage phenotype in adipose tissue.

Abbreviations: AMPKα, AMP-activated protein kinase α; CRP, C-reactive protein; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; HFP, high-fat palm-oil diet; HFPn3, high-fat palm-oil diet supplemented with n3 fatty acids; HOMA-IR, homeostasis model of assessment–insulin resistance; LFC, low-fat control diet; PKB, protein kinase B; PUFA, polyunsaturated fatty acids.

Obesity is accompanied by chronic inflammation in adipose tissue; increased circulating concentrations of TNFα, IL6, and C-reactive protein (CRP); and decreased concentrations of adiponectin.[2] This chronic inflammation links obesity and the development of insulin resistance.[39] Dietary saturated fatty acids promote obesity in part through the induction of inflammation via activation of toll-like receptor 4 (the innate immune receptor for LPS).[28] The absence of functional tlr4 in mice reduces circulating proinflammatory cytokine concentrations and decreases macrophage infiltration into adipose tissue during high-fat diet-induced obesity.[8,28,32] Furthermore, in 3T3 L1 mouse adipocytes, palmitate activates NFκB, protein kinase C, and mitogen-activated protein kinase, all of which increase the production of inflammatory cytokines.[1]

For people who consume a diet high in saturated fat, a major determinant of health is the ratio of omega-6 to omega-3 fatty acids (that is, n6:n3) that is consumed.[5,6] Unlike saturated fatty acids, the n3 polyunsaturated fatty acids (PUFA) eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) exert predominantly antiinflammatory effects, as is evident in that DHA antagonizes NFκB activation by palmitate in 3T3 L1 adipocytes.[1] In mice, EPA prevents or reverses hyperinsulinemia, hyperglycemia, and increased circulating monocyte chemotatic protein 1[16] and decreases infiltration of adipose tissue with macrophages.[30] Moreover, n3 PUFA alleviate the decline in serum adiponectin that is associated with obesity,[12,15,30] and EPA decreases serum CRP in diabetic patients.[26]

Physiologic differences between rodents and humans underscore the need for comparative models in biomedical research, and the pig is emerging rapidly as a model for studies of energy metabolism and obesity. Like humans, pigs are natural omnivores, rely on apolipoprotein B100 to shuttle cholesterol in the LDL fraction, and have minimal brown fat retention postnatally. Furthermore, adipose depots in pigs are of sufficient size that multiple assays can be done on adipocytes or stromal vascular cells without pooling across depots or animals. Although Ossabaw swine have been used as models for metabolic syndrome, cardiovascular disease, coronary artery disease, and steatohepatisis,[11,20,24] little is known about adipose inflammation in these animals. Consequently, we sought to characterize obesity-linked inflammatory markers in the adipose tissue of this novel model and to test the hypothesis that adding n3 PUFA to a diet high in saturated fat attenuates chronic inflammation, protects against diet-induced insulin resistance, and alters phenotypic changes in adipose tissue macrophages.

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Materials and Methods

Animals and diets. All animal procedures were conducted at Iowa State University (Ames, IA) and were approved by its IACUC. Two groups (27 total) of mature male and female Ossabaw

swine (*Sus scrofa*; initial body weight, 47.4 ± 0.3 kg) were housed in individual pens in a temperature-controlled environment with a 12:12-h light:dark cycle. Swine were obtained from the Comparative Medicine Program at the Indiana University School of Medicine and Purdue University from a herd that tested negative for *Brucella* spp. and pseudorabies and showed no clinical evidence of communicable disease on arrival or during the study. Pigs each were assigned to 1 of 3 dietary treatments: a low-fat control (LFC) diet (n = 9; 3 male and 6 female), a high-fat palm-oil (HFP) diet (n = 9; 2 male and 7 female), and a high-fat palm-oil diet supplemented with n3 (HFPn3, n = 9; 2 male and 7 female; Figure 1). The HFP and HFPn3 diets were fed ad libitum, and feeding of the LFC diet was restricted to maintain a mature lean body weight. Body weight and feed intake were measured weekly. The healthy control diet (Figure 1) was designed to limit caloric intake and provide adequate n3 fatty acids through modest inclusions of fish meal, canola oil, and n3 fatty acid supplement (Gromega 365, JBS United, Sheridan, IN). Both high-fat diets were high in saturated fatty acids and formulated to be isocaloric. Canola oil and the n3 fatty-acid supplement were used to reduce the n6:n3 to levels comparable with that of the control diet. To confirm the fatty-acid profile of each diet, lipids were extracted as described previously.[21] Fatty-acid methyl esters were analyzed by gas chromatography (model 6890, Hewlett-Packard, Palo Alto, CA) fitted with an Omegawax 320 (30 m × 0.32 mm internal diameter; 0.25 µm film) capillary column (Sigma–Aldrich, St Louis, MO). The injector and detector temperatures were 250 °C, and the oven temperature was 200 °C.

Insulin challenge and tissue collection. After 30 wk of dietary treatment, pigs were fasted for 12 h overnight and anesthetized with telazol:ketamine:xylazine (1:1:1). Thereafter, blood pressure and mean arterial pressure were measured by using an oxillometric blood pressure monitor (Cardell, CAS Medical Systems, Branford, CT) with a 9-cm cuff on the metatarsus. Each parameter was measured 3 times and averaged. Blood was collected via jugular venipuncture, and then animals were challenged with 0.25 IU/kg of porcine insulin (MP Biomedicals, Solon, CA) intravenously as adapted from a previous publication.[37] Glucose measurements were taken from blood obtained by ear pricks at 0, 5, 10, and 15 min after insulin challenge by using a glucometer (LifeScan, Milpitas, CA). Pigs then were euthanized by exsanguination. Subcutaneous adipose tissue from back fat above the 10th rib, visceral adipose tissue from the ventral intraabdominal cavity, omental adipose tissue, liver, and longissimus dorsi from above the 10th rib were collected. Liver and longissimus dorsi were snap-frozen in liquid nitrogen. Adipose tissue was divided 2 ways: snap frozen in liquid nitrogen or stored in ice-cold PBS with 0.2% BSA for transport to the laboratory and the isolation of stromal vascular cells.

Stromal vascular cells were isolated from adipose tissue as follows: adipose tissue was minced finely with a razor blade and digested in HBSS containing 20 mM HEPES (pH 7.4), 3% BSA, and 2 mg/mL collagenase type I (Worthington Biochemical, Lakewood, NJ) for 45 min at 37 °C with shaking at 125 rotations per minute. Samples then were centrifuged for 5 min at 1000 × g, and the supernatant was discarded. The pellet was dissolved in 10 mL of RBC lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min to lyse RBC, and then 10 mL PBS was added, and samples were passed through a 100-µm filter. The filtrate was centrifuged for 5 min at 1000 × g, and the pellet was resuspended in HBSS with 20 mM HEPES (pH 7.4).

	Diet		
Ingredient	LFC	HFP	HFPn3
Corn	46.92	25.06	13.32
Soybean meal 48	4.00	19.55	21.35
Sucrose	0	28.00	28.00
Palm oil	0	18.00	18.00
Menhaden meal	7.50	0	0
Soybean hulls	33.44	0	0
Wheat bran	3.00	0	0
Canola oil	1.00	0	3.00
Gromega 365[a]	1.00	0	5.00
Meat and bone meal[b]	0	6.25	6.25
Vitamin mix	0.50	0.50	0.50
Salt	0.40	0.40	0.40
Mineral mix	0.10	0.10	0.10
Selenium mix	0.05	0.05	0.05
DL-Methionine	0	0.07	0.09
Dicalcium phosphate	1.88	1.63	1.64
Limestone	0.21	0.38	2.30
Ethoxyquin[c]	0.0125	0.0125	0.0125
Metabolizable energy (kcal/kg)	2750	4059	4058
Fat (kcal/kg)	382	1572	1877
Ether extract	4.69	20.27	23.98
Crude protein	14.92	15.14	15.00
Lysine	0.81	0.80	0.83
Methionine	0.29	0.29	0.29
Met + Cys	0.47	0.50	0.50
Crude fiber	13.69	1.39	1.19
Calcium	0.90	1.20	1.93
Available phosphorous	0.60	0.60	0.60

[a]JBS United, Sheridan, IN
[b]Darling International, Des Moines, IA
[c]Novus International, St Charles, MO

Figure 1. Ingredient composition (% except where noted) of the diets as fed

Flow cytometry. Stromal vascular cells (maximum, 1×10^6) were washed in 2 mL FACS buffer (0.1% sodium azide, 0.1% BSA in PBS). Cells were incubated in 10 µL pig serum for 15 min at 4 °C to prevent nonspecific binding. Cells then were incubated with 10 µL antiCD203a IgG_1 (dilution, 1:20; Mybiosource, San Diego, CA) or isotype-matched control antibody (mouse $IgG_1\kappa$; 1:20; eBioscience, San Diego, CA) for 15 min at 4 °C and washed in 2 mL FACS buffer. Cells were next incubated in 10 µL goat antimouse IgG_1–FITC (1:50; Southern Biotech, Birmingham, AL) for 15 min at 4 °C and then washed in 2 mL FACS buffer. Cells were incubated in 10 µL biotinylated antiCD16 (1:50; BD Pharmingen, San Diego, CA) or isotype-matched control (biotinylated mouse $IgG_1\kappa$; 1:50; BD Pharmingen) for 15 min at 4 °C and then washed in 2 mL FACS buffer. Cells then were incubated in 10 µL of streptavidin–peridinin chlorophyll a protein–Cy5.5 (1:50; BD Pharmingen) and 10 µL antiCD14–PE (1:20; Antigenix America, Huntington Station, NY) or isotype-matched control (PE-conjugated mouse $IgG_{2b}\kappa$; 1:20; BD Pharmingen) for 15 min at 4 °C and washed in 2 mL FACS buffer. Cells were fixed in 200 µL 1% paraformaldehyde and stored at 4 °C until analysis. Analysis was performed on a flow cytometer (FACScanto, Becton Dickinson, San Jose, CA), and data were analyzed by using FlowJo software (version 8.5.2, Tree Star, Ashland, OR).

Serum and tissue analyses. Cholesterol, triglycerides, and nonesterified fatty acids were analyzed by using Chol Slides (Ortho Clinical Diagnostics, Rochester, NY), Trig Slides (Ortho Clinical Diagnostics), and NEFA-HR (Wako Diagnostics, Richmond, VA), respectively, on an automated analyzer (Vitros 5.1, Ortho Clinical

Diagnostics). Serum insulin and CRP concentrations were measured by enzyme immunoassay and ELISA (ALPCO, Salem, NH), respectively. Serum TNFα, IL6, IL10, and IL12p40 concentrations were measured by ELISA (R&D Systems, Minneapolis, MN). Homeostasis model of assessment–insulin resistance (HOMA-IR; a measure of insulin resistance) was calculated by using fasting serum insulin (μU/mL) × fasting blood glucose (mg/dL) ÷ 405.[23] Plasma homocysteine concentrations were determined by HPLC as described previously.[36] Total liver and LD lipid content was determined by using a published method.[13]

Whole frozen-tissue samples were pulverized and homogenized in ice-cold buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 5 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 2 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10% glycerol, and 1% Triton X100). Homogenates were mixed by shaking for 45 min at 4 °C and then centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were collected, and the protein concentration was determined by using BCA reagent (Pierce, Rockford, IL).

Samples were added to a reducing loading buffer with a final composition of 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromophenol blue and heated for 10 min at 100 °C. Equal amounts of protein were separated by SDS-PAGE (10% resolving gel), transferred to a nitrocellulose membrane, and probed with either antiadiponectin rabbit polyclonal antibody (1:750; Alpha Diagnostics, San Antonio, TX), antiSer_{473} protein kinase B (PKB; that is, Akt) rabbit polyclonal antibody (1:2000; Cell Signaling Technology, Danvers, MA), antiPKB (1:1000; Cell Signaling Technology), or antiAMP-activated protein kinase (AMPK) α (1:1000; Cell Signaling Technology) overnight at 4 °C. Both the total and phospho-specific PKB antibodies detect all isoforms. The porcine adiponectin antibody was generated against the peptides DQYQDKNVDQASGS and TEKPGALLPVPKGAC. Antiadiponectin was purified from immunized rabbit serum by using affinity chromatography. Membranes then were probed with goat antirabbit IgG conjugated with horseradish peroxidase (1:10,000; Pierce) for 1 h at room temperature. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce) and imaged with the FOTO/Analyst Luminary FX system (Fotodyne, Hartland, WI). Densitometry was performed by using TL100 software (TotalLab, Newcastle, UK). Serum (2 μL) was diluted in reducing loading buffer, heat-denatured, and separated by SDS-PAGE as described above for estimation of serum adiponectin concentrations. Phosphorylation of AMPKα at Thr_{172} was measured by ELISA (Cell Signaling Technology).

Statistical analysis. All statistical procedures outlined were performed by using SAS 9.2 (SAS, Cary, NC). Residuals were analyzed to detect outliers and assess normality by using the PROC Univariate procedure. Right-skewed data were log-transformed for further analysis. ANOVA was performed by using the PROC GLM procedure. Main effects included diet and tissue, where appropriate. Fixed effects included sex, group, and termination date. Fixed effects were used in the model when $P \leq 0.15$ for the fixed effect or interaction with main effect(s). Blood glucose data from the insulin challenge were analyzed by using an autoregression order of one repeated measures. For flow cytometric data, analysis was weighted on live cell number. In addition, the percentage of $CD203a^+$ was used as a covariate for analysis of CD14 and CD16 data. Data are presented are least-squares mean ± SE. A *P* value of 0.05 was used to define statistical significance.

	Diet		
Fatty acid	LFC	HFP	HFPn3
6:0	0.00	0.00	0.00
8:0	0.00	0.00	0.00
10:0	0.66	0.80	0.75
12:0	0.00	1.44	2.12
14:0	2.34	1.06	1.59
14:1	0.00	0.00	0.00
16:0	18.47	43.98	38.85
16:1	2.51	0.00	0.89
18:0	4.08	5.18	4.83
18:1	28.14	36.6	37.58
18:2n6	34.14	10.73	10.49
18:3n6	0.00	0.00	0.00
18:3n3	3.77	0.23	1.03
20:0	0.44	0.00	0.21
20:1	0.66	0.00	0.00
20:2	0.00	0.00	0.00
20:3	0.00	0.00	0.00
20:3n6	0.00	0.00	0.00
20:4n6	0.00	0.00	0.00
20:5n3	2.33	0.00	0.88
22:1	0.00	0.00	0.00
22:2	0.00	0.00	0.00
24:0	0.00	0.00	0.00
22:6n3	2.10	0.00	0.78
Other	0.37	0.00	0.00
Total	100.00	100.00	100.00
Saturated	26.36	52.45	48.35
n3	8.20	0.23	2.69
n6	34.14	10.73	10.49
n6:n3	4.16	47.59	3.91

Figure 2. Dietary fatty acid profile (g/100 g total fatty acid)

Results

Three pigs in the HFPn3 dietary treatment died during the study. Autopsy results revealed no obvious signs of disease or pathognomonic cause of death.

Dietary fatty-acid profiles are presented in Figure 2. Only the LFC and HFPn3 diets contained measurable EPA and DHA, whereas all 3 diets contained α-linolenic acid. The addition of n3 PUFA to the HFPn3 diet decreased the n6:n3 from 47.6 in the HFP diet to 3.9 in the HFPn3 diet. The average calculated n3 PUFA consumption was 0.40, 0, and 7.6 g daily for EPA and 0.36, 0, and 6.7 g daily for DHA for the LFC, HFP, and HFPn3 groups, respectively. To maintain lean mature body weight, caloric intake was limited in the LFC group to an average of 1778 kcal daily compared with approximately 9800 kcal daily for the 2 high-fat diets. Final body weights for the 2 high-fat groups did not differ (Table 1).

To assess the effects of the high-fat diet and n3 supplementation on systemic metabolic disturbances in pigs, we measured insulin, blood glucose, cholesterol, triglycerides, homocysteine, and blood pressure (Table 1). No differences were detected among the LFC, HFP, and HFPn3 groups in regard to fasting serum insulin, cholesterol, triglyceride, and homocysteine concentrations. In contrast, there was a diet-associated effect on serum nonesterified fatty acids: the HFPn3 group had higher ($P = 0.0453$) concentrations of nonesterified fatty acids than did the LFC group, whereas the levels in the HFP group did not differ from those in either the LFC or the HFPn3 group. In addition, consumption of either high-fat diet increased systolic blood pressure, diastolic blood pressure, and mean arterial pressure. Although serum insulin concentrations

Table 1. Growth and metabolic parameters of Ossabaw swine at end of study

Parameter	Diet			*P*
	LFC	HFP	HFPn3	
Body weight (kg)	49.6 ± 4.7[a]	156.7 ± 5.3[b]	154.4 ± 5.8[b]	<0.0001
Insulin (pg/mL)	63.5 ± 8.6	80.3 ± 11.7	76.5 ± 14.7	0.44
Blood glucose (mg/dL)	119.8 ± 14.8[a]	233.5 ± 29.4[b]	221.5 ± 34.4[b]	0.0011
HOMA-IR	0.44 ± 0.12[a]	1.03 ± 0.13[b]	0.97 ± 0.18[b]	0.0121
Cholesterol (mg/dL)	68.4 ± 5.7	90.8 ± 6.4	79.0 ± 6.9	0.06
Triglycerides (mg/dL)	42.0 ± 7.1	61.9 ± 7.4	61.4 ± 8.7	0.11
Liver lipid (%)	4.23 ± 0.18	4.54 ± 0.18	4.58 ± 0.22	0.39
Longissimus dorsi lipid (%)	3.53 ± 1.06	5.90 ± 1.10	5.98 ± 1.29	0.21
Nonesterified fatty acids (mM)	0.57 ± 0.22[a]	1.13 ± 0.23[a,b]	1.46 ± 0.27[b]	0.0453
CRP (μg/mL)	13.7 ± 2.5	20.6 ± 2.6	18.4 ± 3.0	0.17
TNFα (pg/mL)	24.9 ± 3.2	21.6 ± 2.8	15.5 ± 2.3	0.10
Homocysteine (μM)	11.5 ± 5.8	33.0 ± 5.2	26.2 ± 8.1	0.06
Diastolic blood pressure	63.4 ± 7.9[a]	96.3 ± 7.1[b]	102.0 ± 9.0[b]	0.0251
Systolic blood pressure	127.2 ± 6.9[a]	170.1 ± 6.3[b]	178.7 ± 8.1[b]	0.0021
Mean arterial pressure	93.6 ± 8.9[a]	118.0 ± 7.9[b]	134.0 ± 10.2[b]	0.0275

P values indicate differences between values with different lowercase letters in the same row.

did not differ between groups, LFC-fed pigs had lower ($P = 0.0011$) fasting blood glucose levels than did those fed either the HFP or HFPn3 diet. The high fasting blood glucose concentrations of pigs fed the HFP or HFPn3 diets caused increases ($P = 0.0121$) in HOMA-IR as compared with that of the LFC group.

Diet was not associated with significant effects on serum CRP or TNFα (Table 1). However, serum CRP was positively correlated ($r = 0.42716$, $P = 0.0421$) with fasting blood glucose. IL6, IL10, and IL12 were detectable in the sera of only 2 pigs (data not shown). Serum adiponectin was decreased ($P = 0.0410$) by both high-fat diets compared with the control group, but the magnitude of this decrease was attenuated in pigs fed the HFPn3 diet (Figure 3 A). Serum adiponectin was negatively correlated ($r = -0.44268$, $P = 0.0287$) with fasting blood glucose.

To assess whether differences in serum adiponectin were associated with altered AMPK activity, we measured AMPKα phosphorylation in the longissimus dorsi muscle. Both the HFP and HFPn3 groups had decreased ($P = 0.0055$) phosphorylation of AMPKα at Thr_{172} and a decreased ($P = 0.0136$) ratio of Thr_{172} AMPKα to total AMPKα as compared with the LFC group but did not differ in total AMPKα (Figure 3 B through D). Phosphorylation of AMPKα at Thr_{172} was positively correlated ($r = 0.47112$, $P = 0.0233$) with serum adiponectin concentration and negatively correlated ($r = -0.49803$, $P = 0.0156$) with fasting blood glucose. In addition, we determined that diet did not influence ectopic lipid deposition (as represented by percentage lipid) in liver and longissimus dorsi muscle (Table 1).

Insulin sensitivity was assessed by measuring the decrease in blood glucose after insulin challenge. Blood glucose was decreased by 5 min after insulin administration in the LFC group and by 10 min in the group fed the HFPn3 diet (Figure 4). The HFP group was unresponsive to insulin throughout the 15-min challenge period (Figure 4). Tissue differences in insulin signaling were assessed by evaluating phosphorylation of PKB at Ser_{473}. The HFPn3 diet decreased PKB abundance in subcutaneous, visceral, and omental adipose depots (Figure 5 A, D, and G), but Ser_{473} phosphorylation of PKB did not differ among these tissues (Figure 5 B, E, and H). The HFP diet but not the HFPn3 diet decreased the ratio of Ser_{473} PKB to total PKB in the subcutaneous and omental fat depots (Figure 5 C and I) but not the visceral depot (Figure 5 F). There were no diet-associated differences in total PKB or PKB phosphorylation in either liver or longissimus dorsi muscle (data not shown).

Adipose tissue macrophages were evaluated by flow cytometry to determine both the extent of macrophage (CD203a+) infiltration and phenotype. Phenotype was determined by the presence of CD16, an Fcγ receptor, and CD14, the coreceptor for tlr4. Compared with that in the LFC group, the percentage of macrophages in stromal vascular cells was decreased in the HFP and HFPn3 groups, with no difference among adipose depots (Figure 6 A).

Compared with LFC pigs, the HFP and HFPn3 groups had increased numbers of $CD16^+$ macrophages in the adipose tissue, with no difference among the adipose depots (Figure 6 B). There was no difference in $CD16^+CD14^+$ macrophages among dietary treatments; however, the numbers of $CD16^+CD14^+$ macrophages were decreased in the omental compared with the subcutaneous adipose depot (Figure 6 C). In addition, the HFP diet increased the percentage of $CD16^-CD14^+$ macrophages compared with those of the LFC and HFPn3 groups (Figure 6 D) with no differences across adipose depots.

Discussion

Comparative animal models are crucial to understanding the development of human obesity and the chronic inflammation in adipose tissue that contributes to the prediabetic state (that is, metabolic syndrome). Although previous studies[11,24,33,34] have underscored the potential value of Ossabaw pigs as a model for obesity and prediabetes, the current study is the first to address obesity-linked inflammation in adipose tissue of Ossabaw pigs and the potential alleviation of this inflammation by dietary n3 fatty acids.

Whereas others[20] have reported increased circulating concentrations of TNFα in response to diet-induced obesity in Ossabaw swine, we found no diet-related increase in serum concentrations

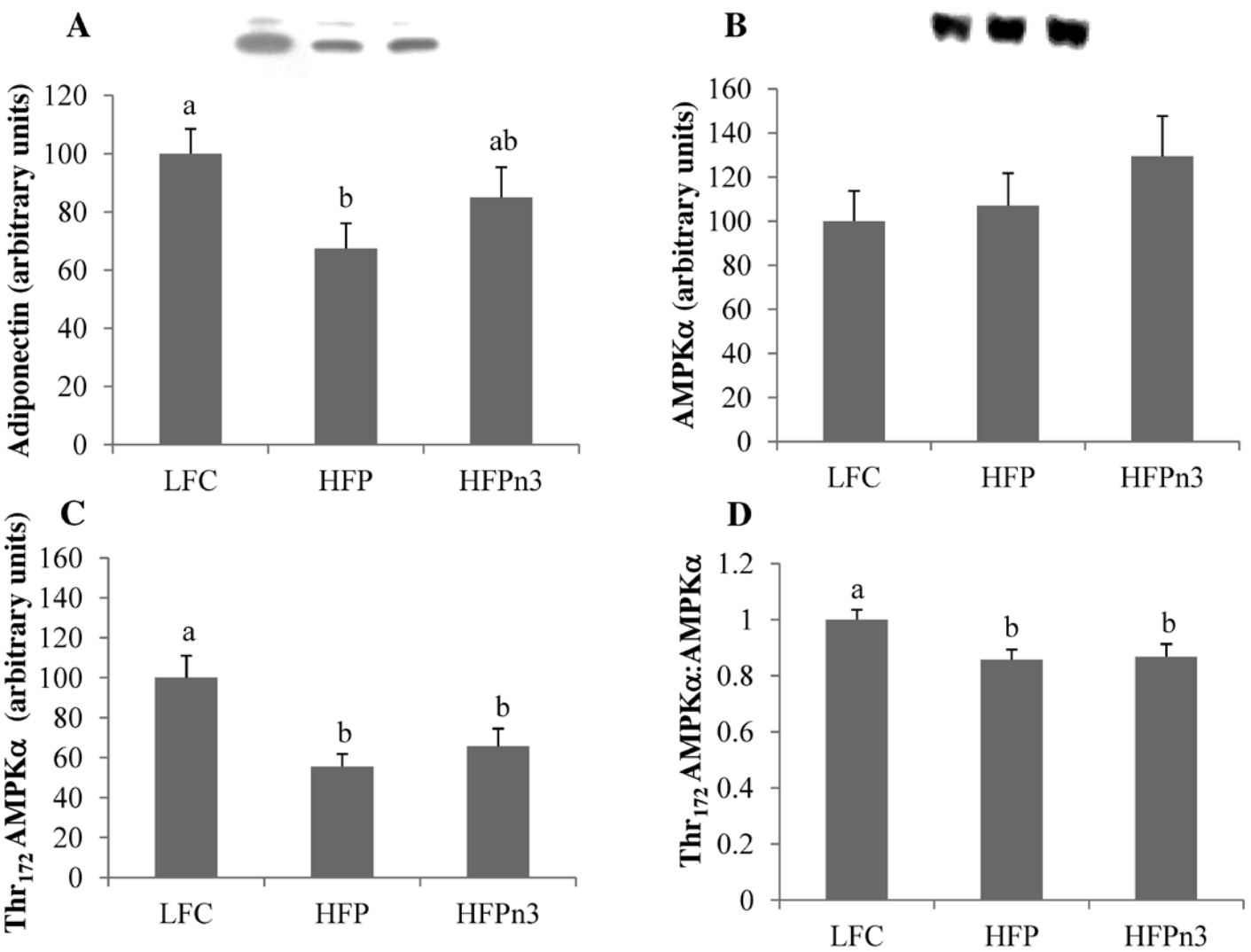

Figure 3. (A) Relative serum adiponectin and (B) AMPKα, (C) Thr_{172}AMPKα, and (D) Thr_{172}AMPKα:AMPKα of LD Ossabaw swine fed the low-fat control (LFC), high-fat palm oil (HFP), or high-fat palm oil plus n3 fatty acids (HFPn3) diet. Gel bands are shown above their associated bars on the respective graphs. Different lowercase letters indicate significant ($P < 0.05$) differences. Main effects: (A) diet, $P = 0.0410$; (B) diet, $P = 0.42$; (C) diet, $P = 0.0055$; (D) diet, $P = 0.0136$.

of the common proinflammatory markers TNFα, CRP, IL6, and IL12, despite the prolonged duration of our study and the marked obesity that developed (that is, more than 100 kg gained beyond typical mature body weight). Whether this discrepancy reflects differences in diet composition across studies is unresolved currently. Others[20] have attributed both inflammation and liver injury to the presence of fructose in atherogenic diets. However, the diet we used contained sucrose, which contributed 14% fructose to the diet on a per-weight basis with no added cholesterol. Reduced ectopic storage of lipid in liver and skeletal muscle due to increased capacity for expansion of adipose mass has been associated with improvements in metabolic markers associated with obesity,[17] and presumably, alleviation of obesity-linked inflammation. We saw no evidence of ectopic lipid accumulation in the liver or skeletal muscle of pigs fed either high-fat diet. Consequently, the marked capacity of the Ossabaw swine to expand adipose mass may, in fact, afford them some protection against obesity-linked inflammation.

To evaluate adipose inflammation in this swine model and make comparisons with data in the human literature, we investigated the macrophage populations in the adipose tissue of lean compared with obese Ossabaw swine. Several studies of human adipose tissue have categorized macrophages as being $CD14^+$.[4,18,35,41] In our study, only a small percentage (8% to 10%) of the adipose-derived stromal vascular cells were identified as being $CD14^+$. Consequently, we used the mature macrophage marker, CD203a, to identify macrophage populations and the markers CD14 and CD16 to assess phenotypic changes associated with diet or adipose depot. Surprisingly, both the HFP and HFPn3 groups had fewer macrophages than did the LFC pigs, although this decrease did not occur in all depots across diets. It seems possible that accelerated macrophage turnover in obese pigs led to an increase in adipose monocytes not expressing CD203a. Recruitment of adipogenic progenitors, due to rapid expansion of adipose mass in pigs fed the high-fat diets, may have contributed

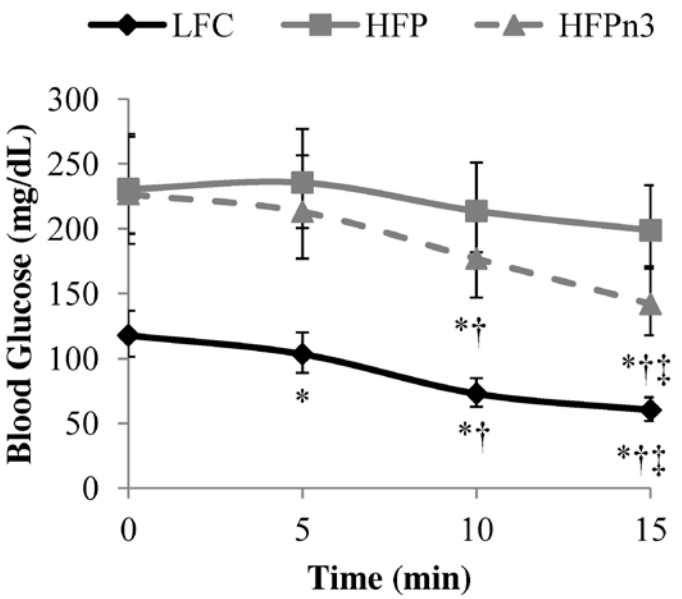

Figure 4. Blood glucose from Ossabaw swine challenged with 0.25 IU/kg porcine insulin after a 12-h fast from 3 dietary treatments: low-fat control (LFC), high-fat palm oil (HFP), high-fat palm oil plus n3 fatty acids (HFPn3). Significant ($P < 0.05$) differences from 0 to 5 min, 5 to 10 min, and 10 to 15 min within diet are represented by *, †, and ‡, respectively. Main effects: diet, $P = 0.0004$; time, $P \leq 0.0001$; diet×time, $P = 0.0481$.

to a reduction in the overall percentage of macrophages in the stromal vascular cell population.

The predominant population of adipose macrophages in our Ossabaw pigs was $CD16^+$, with the vast majority of those cells also $CD14^-$. $CD16^+CD14^-$ monocytes are antiinflammatory.[7] Therefore, adipose inflammation in obese Ossabaw swine may be minimized because the majority of macrophages are of the $CD16^+CD14^-$ phenotype. However, the number of $CD16^-CD14^+$ macrophages was increased in the adipose tissue of pigs fed the HFP diet compared with the LFC or HFPn3 diet. In humans, classic $CD16^-CD14^+$ blood monocytes express high levels of CCR2 and CD62L[29] and thus are proinflammatory.[7] This outcome perhaps indicates that the greater number of $CD16^-CD14^+$ macrophages in the adipose tissue of pigs fed the HFP diet reflects obesity-linked infiltration with proinflammatory cells and that the HFPn3 diet protected against this infiltration, despite the marked obesity. We previously demonstrated that dietary supplementation with n3 fatty acids enriched both adipose tissue and muscle;[14] higher concentrations of n3 PUFA in the adipose tissue of pigs fed the HFPn3 diet may therefore have protected them from the increase in $CD16^-CD14^+$ macrophages.

Regarding indicators of prediabetes, fasting hyperglycemia was readily induced by both high-fat diets, as seen through increased HOMA-IR and blood pressure measures. Hyperglycemia and hyperinsulinemia have been reported[19,20] in pig models, although insulin peaked at 1 mo and decreased thereafter in one study.[19] The degree of hyperglycemia induced in the current study by the high-fat diets without concomitant hyperinsulinemia is striking. Hypoinsulinemia can be present in a diabetic state[9] and may be caused by decreased β-cell function due to chronic hyperglycemia.[10] However, this situation has not been addressed in this swine model, to our knowledge. Although high-fat diets have induced hypercholesterolemia and hypertriglyceridemia in several studies using swine,[11,19,20,24] the diets used in our current study did not cause significant increases in either. We did not add cholesterol to the diets, as was done in other studies.[11,20,24] In Ossabaw swine, dietary cholesterol likely plays a pivotal role in the development of hypercholesterolemia and hypertriglyceridemia. Circulating nonesterified fatty acids were increased in only in the HFPn3 group compared with the LFC group. This result is most likely a consequence of the slightly higher fat content of the HFPn3 diet due to addition of the n3 fatty acid source.

In the context of obesity, the activity of AMPK is quite important because of its stimulation of glucose uptake and fatty acid oxidation in muscle.[31,40] Relative to the control group, we found that both obese groups had decreased abundance of phosphorylated Thr_{172} AMPKα and a decrease in the ratio of phosphoThr_{172} AMPKα to total AMPKα in the longissimus dorsi muscle. Genetically, Ossabaw swine contain the mutation $Val_{199}Ile$ in the PRKAG3 (the γ_3 isoform of AMPK) gene, which negatively affects the activity of AMPK.[22] It has been hypothesized that decreased AMPK activity due to this mutation is causal in the development of insulin resistance in Ossabaw pigs.[27] Although adiponectin clearly stimulates activation of AMPK,[40] it seems unlikely that the relatively small decrease in serum adiponectin in the obese groups compared with the control group contributed to the reduction in Thr_{172} AMPKα. Given the PRKAG3 mutation, it will indeed be intriguing to determine whether Ossabaw pigs are more susceptible to the effects of high-fat diets on AMPK activity than are pigs of commercial lean lines.

Changes in blood glucose concentrations during the insulin challenge indicated that supplementation with n3 fatty acids improved insulin sensitivity compared with that in pigs fed the HFP diet. However, there was no diet-associated difference in Ser_{473}PKB in adipose tissue, liver, or longissimus dorsi muscle after insulin stimulation. In the HFPn3 pigs, total PKB was decreased in all adipose depots compared with that of the LFC pigs, and Ser_{473}PKB:PKB was increased in subcutaneous and omental adipose depots compared with that of the pigs fed the HFP diet. In adipocytes, activation of peroxisome proliferator-activated receptor γ by thiazolidinediones decreases PKBα abundance specifically.[38] Previous work has shown that n3 fatty acids (or their metabolites) are ligand activators of peroxisome proliferator-activated receptor γ,[3,25] and the reduction in PKB in the HFPn3-fed pigs may reflect this activity of n3 fatty acids in adipocytes. Nonetheless, the reduction in PKB did not preclude the stimulation of glucose uptake by insulin during the insulin challenge. In addition, the insulin challenge was performed under anesthesia, which although necessary for this study, is also a limitation.

In summary, we have shown marked obesity, hyperglycemia, and hypertension and a modest reduction in circulating adiponectin concentrations in Ossabaw swine fed a diet high in saturated fat. However, there were only limited indications of the expected obesity-linked inflammation in adipose tissue. The predominate evidence of inflammation was the increased $CD16^-CD14^+$ macrophages in the adipose tissue of the HFP group, which are most likely proinflammatory macrophages. Despite the lack of a clear inflammatory response, the HFP group was less sensitive to exogenous insulin, as compared with the control and HFPn3 groups. The primary effects of n3 fatty

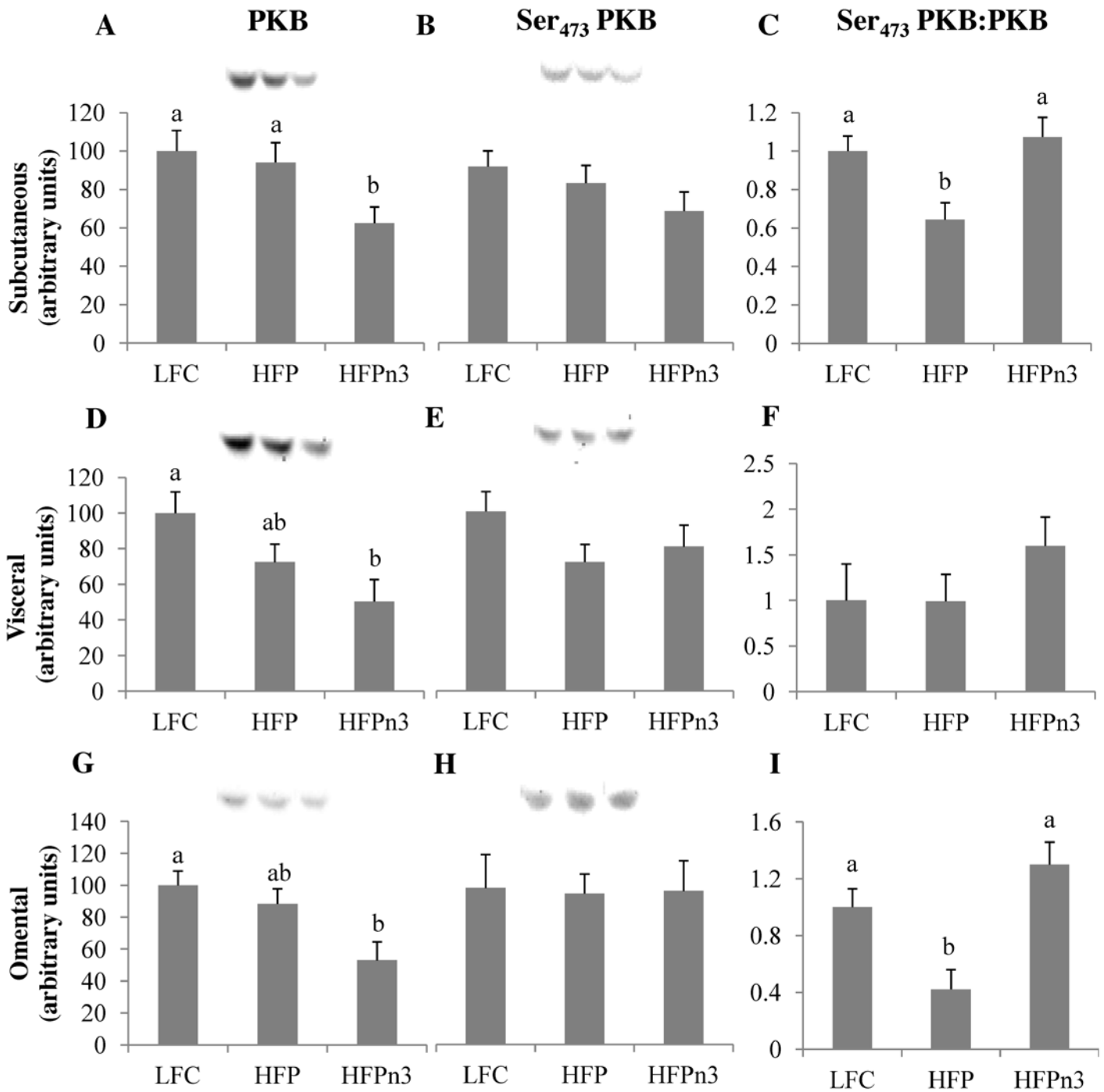

Figure 5. (A) Protein kinase B (PKB), (B) Ser_{473}PKB, and (C) Ser_{473}PKB:PKB of subcutaneous adipose; (D) PKB, (E) Ser_{473}PKB, and (F) Ser_{473}PKB:PKB of visceral adipose (VIS), and (G) PKB, (H) Ser_{473} PKB, and (I) Ser_{473} PKB:PKB of omental adipose (OM) from Ossabaw swine fed low-fat control (LFC), high-fat palm oil (HFP), or high-fat palm oil plus n3 fatty acids (HFPn3) diet. Gel bands are shown above their associated bars on the respective graphs. Different lowercase letters indicate significant ($P < 0.05$) differences. Main effects: (A) diet, $P = 0.0238$; (B) diet, $P = 0.34$; (C) diet, $P = 0.0141$; (D) diet, $P = 0.0326$; (E) diet, = 0.18; (F) diet, $P = 0.22$; (G) diet, $P = 0.0153$; (H) diet, $P = 0.12$; (I) diet, $P = 0.0032$.

acid supplementation were the regulation of PKB abundance, improved insulin-stimulated glucose clearance during the insulin challenge, and blunted infiltration of adipose tissue with $CD16^{-}$-$CD14^{+}$ macrophages. Further investigation with the monocytic markers CD172a and CD163 may provide further insight to adipose depot macrophage populations in this novel model of obesity and prediabetes. Finally, the loss of 3 pigs from the HFPn3 dietary treatment, although not definitively linked to diet, limited our statistical power in studying the beneficial effects of n3 PUFA in this model.

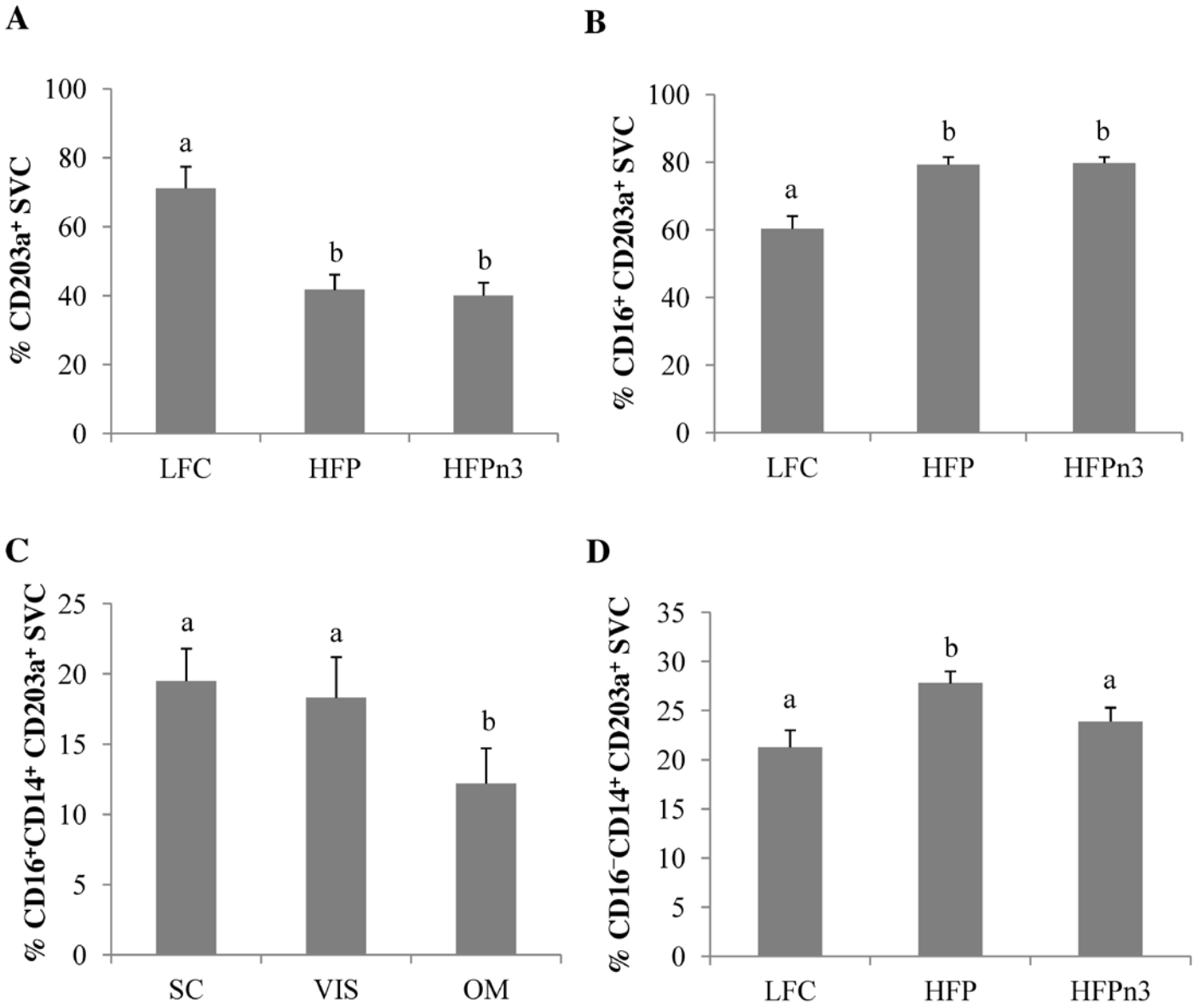

Figure 6. (A) Macrophages (CD203a$^+$) by diet, (B) CD16$^+$ macrophages by diet, (C) CD16$^+$CD14$^+$ macrophages by adipose depot, and (D) CD16$^-$CD14$^+$ macrophages by diet in subcutaneous (SC), visceral (VIS), and omental (OM) adipose depots from Ossabaw swine fed low-fat control (LFC), high-fat palm oil (HFP), or high-fat palm oil plus n3 fatty acids (HFPn3) diet. Data are given as percentage positive cells among (A) total stromal vascular cells (SVC) or (B through D) among macrophages. Different lowercase letters indicate significant ($P < 0.05$) differences. Main effects: (A) diet, $P = 0.0002$; tissue, $P = 0.9192$; diet×tissue, $P = 0.0007$; (B) diet, $P < 0.0001$; tissue, $P = 0.10$; diet×tissue, $P = 0.21$; (C) diet, $P = 0.50$; tissue, $P < 0.0443$; diet×tissue, $P = 0.98$; (D) diet, $P = 0.0053$; tissue, $P = 0.65$; diet×tissue, $P = 0.74$.

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