Materials and Methods

Animals, diets, and tissue collection

To investigate the PPP and NOX4 activity in adipose tissue and subsequent insulin sensitivity during the development of obesity, we performed a time course study in 10 week-old male C57BL/6 mice fed either a high fat, high sucrose (HFHS, 35.5% calories as fat and 36.6% as carbohydrate, 0.15% added cholesterol, BioServ No.F4997) or chow (control) diet for up to 24 weeks which results in obesity, insulin resistance, and both adipose tissue and systemic inflammation [1] (n= 5-6 per group). We have used male mice, because they are more prone than females to the development of visceral obesity, adipose tissue inflammation and insulin resistance in response to a high-fat diet [2-5]. At each of the indicated time points we isolated the pelleted stromal vascular cells (SVC) and the floating cells, i.e., the adipocyte-enriched (AE) fraction, using collagenase (Gibco) treatment of epididymal white adipose tissue (EWAT) [6]. Some mice (fasted for 6h) were injected with saline or insulin (0.2mg) intraperitoneally (i.p.) 15 min prior to sacrifice to evaluate phosphorylated Akt in the AE fraction as a measure of insulin responsiveness [7].

To investigate the pathophysiological role of adipocyte-derived NOX4 in an obesogenic diet-induced obese model, we have crossed an adiponectin-Cre transgenic mouse on a C57BL/6 background (Adipoq-Cre, a kind gift from Dr Philip Scherer, UT Southwestern) with a NOX4 floxed mouse ($NOX4^{Flox/Flox}$, a kind gift from Dr. Sadoshima, University of Medicine and Dentistry of New Jersey) on a C57BL/6 background to obtain the Adipoq-Cre/+;$NOX4^{Flox/Flox}$ mice. Ten week old males of each genotype [Adipoq-Cre/+;$NOX4^{Flox/Flox}$ and Adipoq-Cre/+;$NOX4^{+/+}$ (controls)] were fed either a HFHS or a chow diet for the indicated time points (n= 5-6 per group). Body weights were measured weekly. Intra-peritoneal glucose (GTT) and insulin (ITT) tolerance tests were performed after a 4 h fast as described previously [1], with insulin measured at 30 minutes during the GTT. At euthanasia, harvested tissues were snap-frozen in liquid nitrogen and stored at −70°C or were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All experimental procedures were undertaken with approval from the Institution Animal Care and Use Committee of the University of Washington.

Measurement of NOX, PPP activity, and fatty acid oxidation

AE fractions from EWAT at the indicated time points were collected as previously described [8-10]. NOX activity was measured from the AE fraction by SOD-inhibitable cytochrome C reduction as described previously [11]. To measure PPP activity and β-oxidation, we used [1-^{14}C] glucose, [6-^{14}C] glucose, [1-^{14}C] oleate or [1-^{14}C] palmitate (American Radiolabeled Chemicals Inc.) as previously described [11]. Isolated AE fractions were suspended in Krebs-Ringer buffer containing 1nM insulin and placed in vials. Wells containing cylinders of Whatman filter paper were suspended above the adipocyte-containing solution in vials. The vials were stoppered with rubber caps, gassed with 95% O_2-5% CO_2, and 0.5 μCi of [1-^{14}C, 6-^{14}C] glucose for glucose oxidation, or [1-^{14}C] oleate or [1-^{14}C] palmitate for β-oxidation were injected into the vials. After incubation for 60 min at 37°C, 200μl hyamine hydroxide was injected into the wells containing the filter paper, 200μl of 1N HCl was added to the adipocytes, and the vials were incubated overnight at 37°C. The filter paper in the wells was then transferred to 10ml of scintillation fluid and counted. Using this method, metabolized CO_2 from [1-^{14}C, 6-^{14}C] glucose, [1-^{14}C] oleate or [1-^{14}C] palmitate was successfully trapped in hyamine-soaked filter papers. In the PPP, G6PD and 6-phosphogluconate dehydrogenase (6PGD) make NADPH and CO_2, which is derived from the first carbon of glucose. During glucose metabolism the first carbon only releases CO_2 from the PPP and the Krebs cycle. The Krebs cycle uses all carbons equivalently; therefore, CO_2 released from the 6-carbon of glucose will be the same as CO_2 from the 1-carbon of glucose. Thus, PPP activity can be measured by subtracting CO_2 labeled with 6-^{14}C (which is derived from the Krebs cycle) from CO_2

labeled with 1-^{14}C (derived from both the Krebs cycle and the PPP).

Western blot analysis

Proteins of AE and SVC fractions isolated from indicated time points were separated in 10–20% gradient SDS-PAGE for Western blot analysis using anti–phosphorylated Akt (serine 473), anti-Akt rabbit polyclonal antibodies (Cell Signaling), an anti-F4/80 antibody (ABCAM), an ant-GAPDH antibody (Cell Signaling) and an anti-NOX4 antibody (ABCAM).

Plasma analyses

Plasma was collected at the indicated time points following a 4-hour fast. Insulin and adiponectin were quantified using commercially available ELISA kits (EMD Millipore). SAA, total cholesterol and triglycerides were measured using ELISA (SAA) and colorimetric assays, as described previously [12].

Adipocyte differentiation from pre-adipocytes

Pre-adipocytes were isolated from the stromal vascular fraction of collagenase-digested EWAT from Adipoq-Cre/+;NOX4$^{Flox/Flox}$ and Adipoq-Cre/+;NOX4$^{+/+}$ mice, grown to confluency, and differentiated in DMEM media containing 32 μmol/L dexamethazone, 780 μmol/L 3-isobutyl-1-methylxanthine, 10 μg/ml bovine insulin, 1 μmol/L rosiglitazone and 1 μmol/L Indomethacin for 4 days. Differentiated adipocytes were cultured for 7 days with daily replenishment of DMEM media. To confirm the extent of adipocyte differentiation from pre-adipocytes, total neutral lipid content of cells was visualized by staining with HCS LipidTOX (Invitrogen) and photographed using Image Pro Plus 6.0 (Media Cybernetics).

Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from EWAT and liver tissue and 2 μg reverse-transcribed into cDNA. RT-PCR was performed using the TaqMan Master kit (Applied Biosystems) in the ABI prism 7900HT system [1, 13]. *Saa3*, *Ccl2*, *Tnfα*, *Il1β*, *Il6*, *Cd11b*, *Cd11c*, *Emr1*, *Mac2*, *Nox4, Nox2*, *Apoa1, Pparγ*, *C/ebpα, Pref-1* and *Gapdh* primers with FAM probes were obtained from Applied Biosystems (Assay-on-Demand). Each sample was analyzed in triplicate and normalized using GAPDH as control. Some samples also were normalized with a second housekeeping gene, β-2-microglobulin (*B2m*) and showed similar results.

To quantify mitochondria abundance, the mitochondrial DNA was quantified using qPCR[14]. Genomic DNA from EWAT was extracted by using DNAzol. Total DNA was quantified using the Nanodrop 2000c Spectrophotometer (Thermofisher Sci.). Genomic DNA (100 ng) per reaction was used to perform qPCR. The mitochondrial and nuclear genes primer set were obtained from EMD Millipore. No changes in nuclear DNA levels were observed in any of the experiments.

Preparation of palmitate–albumin complexes

Palmitate (16:0) (Sigma) was conjugated with albumin, as described previously [15]. Briefly, palmitate was first dissolved in NaOH (100 mmol/L) and conjugated with fatty acid-free albumin (Sigma) at a molar ration of 3:1 (palmitate/albumin).

Immunohistochemistry

For immunohistochemistry, EWAT was fixed in 10% formalin for immunohistochemical staining with a Mac2 antibody (1:2,500 dilution, Cedarlane Laboratories), a 4-HNE antibody (1:800 dilution, Alpha Diagnostic International Inc) and a perilipin-I antibody (1:1000 dilution, ABCAM), and photographed as described previously [16]. Area quantification for Mac2 and 4-HNE staining was performed on digital images of immunostained tissue sections using Image Pro Plus 6.0 (Media Cybernetics).

Statistical analysis

Statistical significance was determined with SPSS (Windows version 19) or OriginPro software (version 8.6; Origin Laboratory). All *in vitro* data are shown as means ± SD of three independent experiments performed in triplicate. Student t-test was used to detect differences within groups when applicable (2-tailed and unpaired). One-way ANOVA (ANOVA) was used to compare differences among all groups, and Bonferroni post-hoc testing was used to detect differences among mean values of the groups. $P<0.05$ was considered statistically significant.

REFERENCES

1. Subramanian S, Han CY, Chiba T, McMillen TS, Wang SA, Haw A, 3rd, Kirk EA, O'Brien KD, Chait A. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese ldl receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2008;28:685-691
2. Grove KL, Fried SK, Greenberg AS, Xiao XQ, Clegg DJ. A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice. *Int J Obes (Lond)*. 2010;34:989-1000
3. Stubbins RE, Holcomb VB, Hong J, Núñez NP. Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. *Eur J Nutr*. 2012;51:861-870
4. Stubbins RE, Najjar K, Holcomb VB, Hong J, Núñez NP. Oestrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance. *Diabetes Obes Metab*. 2012;14:58-66
5. Pettersson US, Waldén TB, Carlsson PO, Jansson L, Phillipson M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory t cell population in adipose tissue. *PLoS One*. 2012;7:e46057
6. Montes VN, Turner MS, Subramanian S, Ding Y, Hayden-Ledbetter M, Slater S, Goodspeed L, Wang S, Omer M, Den Hartigh LJ, Averill MM, O'Brien KD, Ledbetter J, Chait A. T cell activation inhibitors reduce cd8+ t cell and pro-inflammatory macrophage accumulation in adipose tissue of obese mice. *PLoS One*. 2013;8:e67709
7. Kim F, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW. Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. *Arterioscler Thromb Vasc Biol*. 2008;28:1982-1988
8. KATZ J, WOOD HG. The use of glucose-c14 for the evaluation of the pathways of glucose metabolism. *J Biol Chem*. 1960;235:2165-2177
9. RODBELL M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem*. 1964;239:375-380
10. Olefsky JM. Mechanisms of decreased insulin responsiveness of large adipocytes. *Endocrinology*. 1977;100:1169-1177
11. Han CY, Umemoto T, Omer M, Den Hartigh LJ, Chiba T, LeBoeuf R, Buller CL, Sweet IR, Pennathur S, Abel ED, Chait A. Nadph oxidase-derived reactive oxygen species increases expression of monocyte chemotactic factor genes in cultured adipocytes. *J Biol Chem*. 2012;287:10379-10393
12. den Hartigh LJ, Wang S, Goodspeed L, Ding Y, Averill M, Subramanian S, Wietecha T, O'Brien KD, Chait A. Deletion of serum amyloid a3 improves high fat high sucrose diet-induced adipose tissue inflammation and hyperlipidemia in female mice. *PLoS One*. 2014;9:e108564
13. Han CY, Chiba T, Campbell JS, Fausto N, Chaisson M, Orasanu G, Plutzky J, Chait A. Reciprocal and coordinate regulation of serum amyloid a versus apolipoprotein a-i and paraoxonase-1 by inflammation in murine hepatocytes. *Arterioscler Thromb Vasc Biol*. 2006;26:1806-1813
14. Tripathy S, Chapman JD, Han CY, Hogarth CA, Arnold SL, Onken J, Kent T, Goodlett DR, Isoherranen N. All-trans-retinoic acid enhances mitochondrial function in models of human liver. *Mol Pharmacol*. 2016;89:560-574
15. Han CY, Kargi AY, Omer M, Chan CK, Wabitsch M, O'Brien KD, Wight TN, Chait A. Differential effect of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: Dissociation of adipocyte hypertrophy from inflammation. *Diabetes*. 2010;59:386-396

16. O'Brien KD, McDonald TO, Kunjathoor V, Eng K, Knopp EA, Lewis K, Lopez R, Kirk EA, Chait A, Wight TN, deBeer FC, LeBoeuf RC. Serum amyloid a and lipoprotein retention in murine models of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2005;25:785-790