Mice lacking NOX2 are hyperphagic and store fat preferentially in the liver

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INFLAMMATION IS AN IMPORTANT CONTRIBUTOR to the development of insulin resistance and type 2 diabetes mellitus (T2DM) (20). Infiltration of immune cells into metabolic tissues such as adipose tissue (27, 32), skeletal muscle (15–16), liver (37), and pancreas (23) in response to obesity or high-fat feeding has been linked to increased inflammation as well as whole body insulin resistance. In particular, inflammatory macrophages have been implicated in the development of insulin resistance in adipose tissue (33) and skeletal muscle (15), whereas anti-inflammatory macrophages may promote insulin sensitivity (16, 48). The mechanisms by which immune cells promote insulin resistance have not yet been fully elucidated.

Reactive oxygen species (ROS) are produced by the mitochondria as a metabolic by-product as well as by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family. In macrophages, pathogens and other stimuli that activate Toll-like receptors require mitochondrial-derived ROS to activate proinflammatory cytokine production (7, 35, 51), whereas NADPH oxidase activation is required to promote phagocytic activity (3). Both mitochondrial- and NADPH oxidase-derived ROS are required to elicit a proinflammatory response to crystalline particles (13). These findings suggest that the source of ROS may be important for stimulus-specific responses of the immune system (34). Although fatty acid-challenged macrophages become inflammatory, it is unknown whether this response requires NADPH oxidase input. The purpose of the current study was to determine whether NADPH oxidase contributes to macrophage inflammation and subsequent insulin resistance in the context of high-fat feeding in mice.

NOX2 (also known as gp91phox and CYBB) interacts with p22phox, p47phox, p67phox, and p40phox to form the NADPH oxidase complex (12, 36, 47, 49), which transfers electrons from NADPH to molecular oxygen, producing a burst of superoxide (1–2, 40, 45). One of seven isoforms (4–5, 8, 11, 14, 18, 25, 43–44), NOX2 is expressed most abundantly in phagocytes (e.g., macrophages, neutrophils, dendritic cells) (39) and at lower levels in various other tissues, including neurons (42). NOX2 localizes both to the plasma membrane and to intracellular membranes (6). Originally thought to produce only extracellular and phagosomal ROS for the purpose of killing bacteria, it is now known that NOX2 also produces intracellular ROS involved in signaling events (24). We hypothesized that macrophages lacking NOX2 would be less inflammatory and that mice lacking NOX2 would have a lessened tissue inflammation in response to high-fat feeding compared with wild-type (WT) mice, affording protection from the development of fat-induced insulin resistance. To test this hypothesis, NOX2 knockout (NOX2-KO) mice were fed a chow or a high-fat (HF) diet for up to 18 wk, and macrophage inflammation as well as whole body parameters of energy handling and insulin action were measured. Surprisingly, despite reduced macrophage inflammation, NOX2-KO mice were more insulin resistant than WT mice when fed a HF diet, and this was associated with hyperphagia and preferential storage of lipid in liver. This model of whole body NOX2 depletion represents a primary hepatic-linked insulin resistance despite reduced macrophage inflammation and epididymal adipose tissue (EWAT) mass.

MATERIALS AND METHODS

Animal studies. Rodent studies were approved by The Hospital for Sick Children Animal Care Committee. C57BL/J mice lacking the Cybb gene (B6.129S-Cybb<sup>tm1Din/J</sup>) (22) were obtained from The Jackson Laboratory (Bar Harbor, MA, stock no. 002365) and are referred to as NOX2-KO mice throughout the text. WT C57BL/J mice were used as controls (The Jackson Laboratory). Male mice were fed a standard Chow diet (5P07 Prolab RMH 1000; LabDiet, St. Louis, MO) or a HF (60% by kcal) defined diet (D12492; Research Diets, New Brunswick, NJ) for 13 or 18 wk from weaning. Mice were singly caged and maintained at 21–22°C with light from 0600–1800. Cages
containing WT animals and KO animals were interspersed. Body weight and food intake were assessed every week at the same time of day. Fasting blood glucose was measured, and serum was collected after 3, 5, 7, 9, and 11 wk on the diet. Insulin tolerance tests were performed after 5, 10, and 17 wk on the diet and glucose tolerance tests after 12 and 16 wk on the diet. Indirect calorimetry was performed after 13 wk on the diet. Mice were euthanized via cervical dislocation following a 4-h fast after 13 or 18 wk on the diet. Tissues were collected, flash-frozen in liquid nitrogen, and preserved at −80°C for further analysis.

Isolation and culture of bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDM) were isolated as previously described (29), with modifications. Bone marrow was removed from the femur and tibia of chow-fed 6- to 8-wk-old WT and NOX2-KO mice by centrifuging bones at 15,000 g for 10 s. Cells were cultured in RPMI medium (Wisent, Saint-Bruno, QC, Canada) supplemented with 10% fetal bovine serum, 1× nonessential amino acids (Wisent), 1 mM sodium pyruvate (Wisent), 25 μM 2-mercaptoethanol (Life Technologies, Carlsbad, CA), 1× antibiotic-antimycotic (Wisent), and 25 ng/ml macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ) for 7 days with 10 ng/ml interleukin-4 (PeproTech) present during the last 48 h before harvest. BMDM from WT mice were treated with 1 mM apocynin (Sigma-Aldrich, St. Louis, MO) or 100% ethanol vehicle for 6 h. RNA was extracted, cDNA was synthesized, and qPCR was performed as described below.

Gene expression analysis by qPCR. Tissues were flash-frozen in liquid nitrogen immediately following dissection. RNA was extracted using TRiZol (Life Technologies, Carlsbad, CA) and cDNA generated by reverse transcription using the SuperScript VILO cDNA kit (Life Technologies) according to the manufacturer’s instructions. qPCR reactions were run using 5 μg of RNA and predesigned Taqman probes (Life Technologies) on a CFX96 C1000 Touch thermal cycler (Bio-Rad, Mississauga, ON, Canada) using the following parameters: one cycle of 95°C for 20 s, followed by 40 cycles at 95°C for 1 s, and 60°C for 20 s. Gene expression was normalized to that of housekeeping genes Hprt or Eef2 for tissues (28) and Abt1 for macrophages.

Body weight, food intake, and naso-anal length. Mouse body weight and uneaten food were measured on a KHA 601 electronic balance (Kilotech, Lachine, QC, Canada) at 3:00 PM each week of the study. Naso-anal length was measured following cervical dislocation after 13 wk on the diet.

Indirect calorimetry. Oxygen consumption (V\textsubscript{O2}), carbon dioxide production (V\textsubscript{CO2}), and ambulatory activity were measured using a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Energy expenditure was measured over 24 h after a 24-h acclimation period. Respiratory exchange ratio (RER) was calculated as V\textsubscript{CO2}/V\textsubscript{O2}. Ambulatory activity was estimated by the number of infrared beam breaks along the x-axis of the metabolic cage. Data were analyzed using CLAMS eXamination Tool (CLAX; Columbus Instruments) version 2.1.0.

Tissue histology. Histology was performed by the University Health Network Pathology Research Program at the Toronto General Hospital (Toronto, ON, Canada). EWAT was preserved in 10% formalin immediately following dissection. EAT sections were stained with hematoxylin and eosin (H&E) and evaluated with an optical microscope (Nikon Eclipse 80i; Nikon, Melville, NY).

Fig. 1. Bone marrow-derived macrophages (BMDM) of NOX2-KO mice have reduced proinflammatory gene expression. Expression of inducible nitric oxide synthase (Nos2; A) and interleukin-6 (Il6; B) in BMDM from WT mice treated with 1 mM apocynin for 6 h. Expression of Nos2 (C), Il6 (D), and arginase-1 (Arg1; E) in BMDM from WT and NOX2-KO mice fed a chow diet for 6–8 wk. Genes are expressed relative to the housekeeping gene activator of basal transcription 1 (Abt1). Results are expressed as means ± SE; n = 3–4 individual mice. Unpaired Student’s t-test, **P < 0.01, ***P < 0.001.
Glucose levels were measured using an Ultra 2 standard curve, and normalized to protein content, spectrophotometrically at 620 nm relative to an oyster glycogen (NIH, Bethesda, MD) by nicking the tip of the tail. Glucose and insulin tolerance tests. After a 4-h fast, basal blood glucose levels were measured using an Ultra 2 glucometer (One-Touch, Burnaby, BC, Canada) by nicking the tip of the tail. Fasting serum insulin was determined using the leptin mouse ELISA kit (Abcam, Cambridge, MA) as per the manufacturer’s instructions. Western blotting. Liver and quadriceps tissue were homogenized in lysis buffer (20 mM Tris, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 10 mM sodium pyrophosphate, 10 mM Na3VO4, 20 mM NaF, 1 mM dithiothreitol, 5% glycerol, 1% NP-40 and 1× protease inhibitor cocktail (Sigma-Aldrich) using a hand-held homogenizer on ice. Homogenates were centrifuged at 15,000 g. Homogenates were lysed by freeze-thaw followed by shearing through a 25-G needle. Samples were then incubated at 97°C for 20 min in 30% KOH saturated with anhydrous Na2SO4. Glycogen was precipitated with 95% ethanol, dissolved in H2O and incubated at 97°C for 20 min with 0.2% anthrone in H2SO4. Glycogen concentration was determined spectrophotometrically at 620 nm relative to an oyster glycogen standard curve, and normalized to protein content.

Hepatic glycogen. Liver glycogen content was determined as previously described (17), with minor modifications. Liver tissue was homogenized in PBM buffer (20 mM KH2PO4, 10 μM CaCl2, 1 mM MgCl2, pH 6.1) using a hand-held homogenizer (Kimble Chase, Kontes, Vineland, NJ) at a ratio of 1 mg tissue to 5 μl of buffer. Cells were lysed by freeze-thaw followed by shearing through a 25-G needle. Samples were then incubated at 97°C for 20 min in 30% KOH saturated with anhydrous Na2SO4. Glycogen was precipitated with 95% ethanol, dissolved in H2O and incubated at 97°C for 20 min with 0.2% anthrone in H2SO4. Glycogen concentration was determined spectrophotometrically at 620 nm relative to an oyster glycogen standard curve, and normalized to protein content.

Glucose and insulin tolerance tests. After a 4-h fast, basal blood glucose levels were measured using an Ultra 2 glucometer (One-Touch) by nicking the tip of the tail. Mice were given an intraperitoneal (ip) injection of 10% glucose (in 0.9% saline) equivalent to 1 g glucose/kg body wt or an ip injection of 1 U (week 5), 1.25 U (week 10), or 1.5 U (week 17) Humulin R U-100 (Eli Lilly Canada, Toronto, ON, Canada) insulin per kg body weight in 0.9% saline. Blood glucose levels were measured at 15, 30, 45, 60, and 90 min postinjection.

Statistical methods. Data are expressed as means ± SE. Unpaired Student’s t-tests were used to detect differences between means in data sets containing two groups. A two-way ANOVA with Tukey posttest was used to detect differences in data sets containing two variables (i.e., genotype and diet). Statistical significance was set at P < 0.05. Graphs were prepared and data analyzed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

RESULTS

Macrophages from NOX2-KO mice present lower inflammatory indexes than WT mice. When BMDM from WT mice were treated with the NOX2 inhibitor apocynin, expression levels of the inflammatory marker inducible nitric oxide synthase (Nos2) were reduced (Fig. 1A), and there was a trend for decreased interleukin-6 (IL6) expression (Fig. 1B). BMDM from NOX2-KO mice expressed lower levels of Nos2 and IL6 compared with macrophages from WT mice (Fig. 1, C and D) but showed no differences in the anti-inflammatory marker arginase-1 (Arg1) (Fig. 1E). In a pilot study, BMDM from NOX2-KO mice secreted lower levels of chemokine (C-X-C motif) ligand 1 (CXCL1; KC) and interleukin (IL)-6 under resting conditions, 6 and 48 h post-medium change (data not shown). We therefore hypothesized that mice lacking NOX2 would be protected from the development of diet-induced insulin resistance since the macrophages infiltrating the adipose tissue, skeletal muscle, liver, and pancreas in response to high-fat feeding would be less inflammatory than those of WT mice. To test this hypothesis, WT and NOX2-KO mice were fed a chow or a HF (60% by kcal) diet for 13 or 18 wk from...
Weaning and analyzed for indexes of whole body insulin resistance.

NOX2-KO mice are hyperphagic and gain more weight than WT mice. As expected, both WT and NOX2-KO mice fed the HF diet gained more weight than when fed the chow diet; however, unexpectedly, NOX2-KO mice gained significantly more weight than WT mice when fed either the chow or the HF diet (Fig. 2A) but had no differences in naso-anal length (data not shown). NOX2-KO mice were in fact hyperphagic, consistently consuming more food on either the chow or the HF diet (Fig. 2B). Higher body weight in KO mice was one of the earliest characteristics noted, becoming statistically significant after 4 wk on the HF diet and after 8 wk on the chow diet.

NOX2-KO mice display equal energy expenditure and spontaneous physical activity compared with WT mice. Indirect calorimetry was performed after mice had been on the diets for 13 wk. There were no differences in average $V_{O2}$ in NOX2-KO mice compared with WT during the light or the dark cycle (Fig. 3, A–C), indicating similar levels of energy expenditure. There were no genotype differences in total $V_{O2}$ during the light or the dark cycle either (data not shown). There were also no genotype differences in RER or spontaneous physical activity; however, HF-fed mice of both genotypes had lower RERs (Fig. 3, D–F) and lower total (Fig. 3H) and ambulatory (Fig. 3J) x-axis activity compared with those on the chow diet.

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**Fig. 3.** Oxygen consumption ($V_{O2}$), respiratory exchange ratio (RER), and spontaneous physical activity. A–C: $V_{O2}$ and average $V_{O2}$ per kg body wt during light and dark cycles in mice fed a chow or HF diet for 13 wk. D–F: RER and average RER during light and dark cycles. Average total x-axis activity (G and H) and average ambulatory x-axis activity (I and J) during light and dark cycles. Results are expressed as means ± SE; $n = 4–8$ mice per group. Two-way ANOVA, Tukey posttest: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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E1344 NOX2-KO MICE HAVE HEPATIC STEATOSIS

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HF-fed NOX2-KO mice gain less EWAT mass than HF-fed WT mice. On the basis of the increased body weight and food intake results, we hypothesized that NOX2-KO mice would have increased adipose tissue mass compared with WT mice; however, there were no genotype differences in IWAT weights (Table 1), and, most surprisingly, HF-fed NOX2-KO mice had lower EWAT mass than HF-fed WT mice (Fig. 4, A and B). Both WT and NOX2-KO HF-fed animals had significantly larger adipocytes compared with chow-fed animals (Fig. 4, C and D); however, there were no genotype differences in adipocyte size. This indicates that NOX2-KO mice were able to increase their adipocyte size in response to HF feeding despite having lower EWAT mass. Although HF-fed NOX2-KO mice had lower EWAT mass than WT mice, they produced the same amount of serum leptin over time (Fig. 4E). Because leptin is primarily produced by adipocytes, this observation suggests that adipocytes in NOX2-KO mice mounted an overall normal adipokine response compared with those in WT mice.

NOX2-KO mice have overwhelming liver mass and steatosis compared with WT mice. One of the most notable phenotypic characteristics of the NOX2-KO mice was their enlarged hepatic mass. Both chow-fed and HF-fed NOX2-KO mice had enlarged livers compared with corresponding WT mice on either diet (Fig. 5, A and B). In addition, NOX2-KO mice had strikingly higher levels of hepatic lipids after 18 wk on the diets, as indicated by substantially stronger Oil red O staining (Fig. 5D). Chow-fed NOX2-KO mice had more hepatic lipid than chow-fed WT mice, and HF-fed NOX2-KO mice had more hepatic lipid than HF-fed WT mice. In contrast, there were no genotype differences in liver glycogen content, although we observed a reduction in liver glycogen with HF feeding (Fig. 5C). To determine whether there was a defect in hepatic de novo lipogenesis, we measured expression of sterol regulatory element-binding protein-1c (Srebf1; SREBP-1c; Fig. 5E), carbohydrate response element-binding protein (Mlxipl; ChREBP; Fig. 5F), stearoyl-coenzyme A desaturase-1 (Scd1; Fig. 5G), and fatty acid synthase (Fasn; Fig. 5H). SREBP-1c and ChREBP are transcription factors whose activation leads to transcription of genes involved in lipogenesis, such as Scd1 and Fasn. There were no diet or genotype differences in Srebf1; however, there were significant differences in Mlxipl, Scd1, and Fasn. HF-fed WT mice had lower expression of Mlxipl than chow-fed WT mice, but chow-fed NOX2-KO mice had significantly lower expression of Mlxipl than chow-fed WT and showed no further decrease upon HF feeding. Additionally, de novo lipogenesis genes Scd1 and Fasn were lower in NOX2-KO mice compared with WT mice (under HF feeding conditions in the case of Scd1 and under both chow and HF feeding conditions in the case of Fasn). In addition to hepatomegaly, NOX2-KO mice also displayed splenomegaly (Table 1), as reported previously (30).

NOX2-KO mice are more prone to develop fat-induced insulin resistance than WT mice. Although chow-fed NOX2-KO mice showed slightly lower blood glucose over the course of the study (Fig. 6A) as well as better glucose tolerance at 12 wk (data not shown) and 16 wk (Fig. 6C), no genotype

Table 1. Wet tissue weights from WT and NOX2-KO mice fed a chow or HF diet for 13 or 18 wk

| Tissue | Chow 13 Weeks on Diet | | | | Chow 18 Weeks on Diet | | | |
|--------|------------------------|---|---|---|---|---|---|---|---|---|---|---|
|        | WT         | KO | WT         | KO | P value | WT         | KO | WT         | KO | P value |
| Soleus | 13 ± 1     | 19 ± 2 | 15 ± 2 | 17 ± 1 | NS | 16 ± 1 | 21 ± 1 | 21 ± 1 | 22 ± 1 | NS |
| Gastrocnemius | 264 ± 10 | 238 ± 17 | 269 ± 7 | 300 ± 14 | *P < 0.05 | 273 ± 12 | 283 ± 7 | 296 ± 13 | 323 ± 8 | NS |
| Quadriceps | 257 ± 14 | 252 ± 36 | 265 ± 20 | 265 ± 15 | NS | 302 ± 19 | 289 ± 21 | 318 ± 21 | 333 ± 17 | NS |
| IWAT | 355 ± 39 | 472 ± 170 | 1515 ± 272 | 1535 ± 134 | **P < 0.001 | ND | ND | ND | ND | NS |
| BAT | 129 ± 9 | 158 ± 12 | 219 ± 28 | 262 ± 28 | **P < 0.001 | 146 ± 32 | 169 ± 14 | 308 ± 35 | 268 ± 84 | **P < 0.01 |
| Pancreas | 153 ± 11 | 178 ± 18 | 192 ± 23 | 235 ± 19 | NS | 195 ± 25 | 188 ± 22 | 319 ± 30 | 278 ± 12 | **P < 0.01 |
| Heart | 133 ± 3 | 142 ± 5 | 146 ± 5 | 177 ± 5 | **P < 0.001 | 175 ± 11 | 176 ± 13 | 200 ± 12 | 206 ± 7 | NS |
| Kidneys | 361 ± 12 | 377 ± 33 | 395 ± 9 | 431 ± 12 | NS | 385 ± 17 | 457 ± 7 | 450 ± 23 | 496 ± 13 | **P < 0.01 |
| Spleen | 56 ± 3 | 125 ± 20 | 72 ± 6 | 139 ± 12 | **P < 0.001 | 80 ± 5 | 182 ± 31 | 98 ± 5 | 198 ± 14 | **P < 0.01 |
| Brain | ND | ND | ND | ND | ND | 414 ± 13 | 406 ± 17 | 422 ± 6 | 416 ± 7 | NS |

Values are means ± SE in mg; n = 4–8 (13 wk); n = 6–7 (18 wk). WT, wild-type; KO, NOX2 knockout; HF, high-fat diet; IWAT, inguinal white adipose tissue; BAT, brown adipose tissue; NS, not significant; ND, not determined. Two-way ANOVA, Tukey posttest, diet differences: *P < 0.05, **P < 0.01, ***P < 0.001; genotype differences: #P < 0.05, ##P < 0.01, ###P < 0.001.
Fig. 4. Epididymal white adipose tissue (EWAT) mass and function. EWAT wet weight in WT and NOX2-KO mice fed a chow or HF diet for 13 (A) or 18 (B) wk. Quantification (C) and representative images (D) of 13-wk EWAT fat cell size. E: serum leptin over time. Results are expressed as means ± SE; n = 4–8 (A, B, E), n = 2 (C) mice per group. Two-way ANOVA, Tukey posttest: *P < 0.05, **P < 0.01, ***P < 0.001 (A–C); #P < 0.05, ##P < 0.01 Chow vs. HF KO; ***P < 0.001 Chow vs. HF WT (E).
differences in fasting glucose or glucose tolerance were detected in HF-fed mice. HF-fed NOX2-KO mice had higher insulin levels than HF-fed WT mice after 7 wk on the diet (Fig. 6B). There were no differences in insulin tolerance between NOX2-KO mice and WT mice on either diet after 5 or 10 wk (data not shown), but HF-fed NOX2-KO mice were less sensitive to insulin than HF-fed WT mice after 17 wk on the diet (Fig. 6D). After 18 wk on the diet, fasting mice were given a meal at 0800 hr, and blood was collected for measurement of glucose and insulin levels 90 min later. NOX2-KO mice fed a chow diet had lower fasting insulin levels than WT mice (Fig. 6C). NOX2-KO mice had higher liver weight and lipid content, but lower expression of genes involved in de novo lipogenesis. Liver wet weight (A and B), glycogen (C), lipid (Oil red O staining; D), and expression of sterol-regulatory element binding protein-1c (Srebf1; E), carbohydrate response element-binding protein (Mlxipl; F), stearoyl-coenzyme A desaturase-1 (Scd1; G) and fatty acid synthase (Fasn; H) in WT and NOX2-KO mice fed a chow or HF diet. Results are expressed as means ± SE; n = 4–8 (A–C), n = 3 (D), or n = 5–7 (E–H) mice per group. Two-way ANOVA, Tukey posttest: *P < 0.05, **P < 0.01, ***P < 0.001.
an intraperitoneal injection of saline or insulin. Mice were euthanized 20 min postinjection, and insulin-stimulated phosphorylation of Akt was measured in quadriceps muscle (Fig. 6E) and liver (Fig. 6F). There was a clear trend for increased phosphorylation of Akt in response to insulin in both the quadriceps muscle and the liver, but data did not reach significance. Curiously, basal levels of Akt phosphorylation in chow-fed WT mice given a saline injection were higher compared with all other saline-injected groups (Fig. 6E). The most salient finding was that the liver of HF-fed KO mice are insulin resistant according to the pAkt response criterion. Pair-feeding studies will be required to distinguish between the contribution of overnutrition or body weight and the contribution of NOX2 to the development of insulin resistance.

**NOX2-KO mice show abundant hepatic macrophage infiltration and inflammation.** Given that high-fat feeding and insulin resistance are associated with macrophage infiltration in metabolic tissues, we assessed the presence of macrophage markers in EWAT, liver, and quadriceps muscle of NOX2-KO and WT mice. There were no genotype differences in expression of the macrophage marker F4/80 (Emr1) in EWAT or skeletal muscle when we compared mice on equivalent diets (Fig. 7, A and C). In contrast, liver of NOX2-KO mice presented with much higher F4/80 expression than WT mice on
both the chow and HF diets (Fig. 7B). Cd11c (Itgax) is a marker of inflammatory macrophages, and expression of this gene was equally and selectively higher in liver of NOX2-KO compared with WT mice, paralleling the expression of F4/80. (Fig. 7, D–F). Additionally, Cxcl1, Cxcl2, Il6, Il12a, tumor necrosis factor (Tnf), and Nos2, indexes of inflammation, were significantly elevated in the liver of NOX2-KO mice compared with WT, on either chow or HF regimens (Fig. 8, A–F). Moreover, livers of NOX2-KO mice showed a trend toward expressing lower levels of the anti-inflammatory marker Arg1 compared with WT mice (Fig. 8G). Collectively, these data reflect a highly proinflammatory status of the liver in mice lacking NOX2.

HF-fed NOX2-KO mice have lower expression of the anorexigenic hypothalamic neuropeptide Pomc. As no differences were found in serum leptin levels, we assessed the hypothalamic expression of appetite regulatory neuropeptides in an effort to understand differences in food consumption between NOX2-KO and WT mice. In samples derived from isolated hypothalami, there were no obvious differences in gene expression of the anorexigenic neuropeptides Agrp (agouti-related peptide) or Npy (neuropeptide Y) (Fig. 9, A and B) across genotypes. Similarly, there were no differences in expression of the orexigenic neuropeptide Grp (gastrin-releasing protein; Fig. 9D); however, HF-fed WT mice had higher expression levels of the anorexigenic neuropeptide Pomc (propioiomenalocortin) compared with chow-fed mice, but NOX2-KO mice failed to mount this increase in Pomc expression in response to HF-feeding (Fig. 9C). Hence, NOX2-KO mice appeared to lack an appetite-curbing signal that normally arises with overnutrition.

DISCUSSION

This study arose to test the hypotheses that bone marrow-derived macrophages (BMDM) from mice lacking the NOX2 subunit of the NADPH oxidase complex would show a lower inflammatory tone than WT macrophages and that this would be associated with protection from the development of high-fat feeding-induced insulin resistance in NOX2-KO mice due to lower levels of inflammatory macrophages in metabolically relevant tissues. NOX2-KO BMDM were indeed less proinflammatory than WT BMDM; however, and, surprisingly, NOX2-KO mice were not protected from the development of fat-induced insulin resistance; in fact they were more susceptible to it, and this was associated with hyperphagia and excessive hepatic steatosis. This hepatic phenotype was manifested in mice fed a normal chow diet and was exacerbated upon HF feeding. Hence, an overall reduction in BMDM inflammatory tone does not necessarily afford protection from insulin resistance, possibly because NOX2-KO mice had increased hepatic macrophage infiltration. Moreover, this animal model reveals a participation of NOX2 in the preferential accumulation of lipid in the liver (as opposed to EWAT) and an escape from normal appetite-curbing cues.

The B6.129S-Cybb<sup>tm1Din</sup>/J mice used in this study are whole body NOX2-null animals. Although NOX2 is most highly expressed in phagocytes (39), it is expressed at lower levels in many other tissues, including neurons (42). While this precludes tissue attribution of the origins of the whole body phenotype observed, the characteristics of the NOX2-KO mice reveal that the enzyme is normally required for proper control
of food intake. Hence, NOX2-KO mice are hyperphagic and accordingly gained more weight both on the regular chow diet and on the HF diet compared with WT mice. A role for NOX2 in the control of food intake is novel and opens the door to future investigation. NOX2 is expressed by hypothalamic neurons and microglia (of myeloid origin). The cells and mechanisms responsible for overfeeding in NOX2-KO mice should reveal novel interplays in the highly integrated control of food intake. The second notable characteristic of NOX2-KO mice is that they gain substantially more lipid in the liver compared with WT mice on either chow or HF diets and conversely have smaller epididymal adiposity.

While this study was ongoing, Pepping et al. (38) also found that NOX2-KO mice become hyperphagic when on a HF diet, although they failed to see this phenotype in chow-fed mice. Overall, the mice in that study showed less pronounced differences with WT mice compared with those in our study, perhaps because the diets were not fed from weaning but from 4 mo of age. Pepping et al. equally reported differences in food intake but did not examine the energy expenditure of the mice, parameters of hypothalamic appetite-controlling peptides, or hepatic parameters. Our study examined NOX2-KO mice in a more comprehensive fashion and identified that the exacerbated gain in body weight was not compounded by diminished energy expenditure. The hyperphagia observed in the HF-fed NOX2-KO mice may be explained in part by the failure to increase hypothalamic expression of the anorexigenic neuropeptide POMC in response to overnutrition, opening the door for detailed future investigation of the underlying mechanism. In anorexigenic POMC-expressing neurons in the arcuate nucleus.

Fig. 8. NOX2-KO mice have increased hepatic inflammation. Chemokine (C-X-C motif) ligand 1 (Cxcl1; A), chemokine (C-X-C motif) ligand 2 (Cxcl2; B), interleukin-6 (Il6; C), interleukin-12a (Il12a; D), tumor necrosis factor (Tnf; E), inducible nitric oxide synthase (Nos2; F), and arginase-1 (Arg1; G) expression in liver of WT and NOX2-KO mice fed a chow or HF diet for 18 wk. Results are expressed as means ± SE; n = 4–8 per group. Two-way ANOVA, Tukey posttest: *P < 0.05, **P < 0.01, ***P < 0.001.
of the hypothalamus, POMC is cleaved to produce α-melanocyte-stimulating hormone (α-MSH). α-MSH binds to the melanocortin-3 (MC3) and melanocortin-4 (MC4) receptors in the paraventricular nucleus (PVN) and other areas (9–10), resulting in lower food intake and elevated energy expenditure (19, 26, 46). In concert with other neuropeptides such as NPY and AgRP, POMC regulates energy expenditure in both the short term (in response to increased circulating insulin after a meal) and the long term (in response to increased circulating leptin with weight gain). Leptin is an adipokine that is secreted in high amounts when adipose tissue stores are high and in low amounts when adipose tissue stores are low. Leptin activates POMC-expressing neurons to suppress food intake and control energy balance over time. Interestingly, we found no genotype differences in circulating leptin levels, suggesting that NOX2-KO mice had whole body adiposity similar to that of WT mice despite having less EWAT mass. Since NOX2 is expressed in hypothalamic cells, we hypothesize that it is required for proper regulation of the POMC response to leptin. It is important to note that no differences were detected in POMC expression in chow-fed mice, despite the fact that the Chow-fed NOX2-KO mice did in fact have increased food intake. This indicates that POMC can be only partially responsible for the hyperphagia observed in NOX2-KO mice. Future studies will focus on this important area.

Curiously, body weights of HF-fed WT mice “caught up” to those of HF-fed NOX2-KO mice after 17 wk on the diet. The weight curves of the Chow-fed WT and NOX2-KO mice as well as the HF-fed NOX2-KO mice followed the expected shape, gradually coming to a plateau. The weight curve of the HF-fed WT mice followed the same pattern until 16 wk, at which point the HF-fed WT mice seemed to undergo another increase in body weight. We are unable to explain this uncharacteristic surge in body weight seen in the HF-fed WT mice but not in the other groups; however, future studies will confirm whether the increased body weight observed in HF-fed NOX2-KO mice does indeed persist beyond 4 mo of feeding.

The lesser expansion of epididymal adipose mass of NOX2-KO mice despite their hyperphagia and increased body weight relative to WT mice deserves comment. The alteration in adipose tissue was depot specific, as we found no genotype-associated differences in subcutaneous adipose mass; however, and strikingly, NOX2-KO mice did not gain nearly as much epididymal adipose mass as WT mice when challenged with a HF diet. This feature was also observed by Pepping et al. (38), but, unlike their findings, the adipocytes of the NOX2-KO mice studied in our study were able to increase their size in response to overnutrition. Moreover, their leptin production was not altered. These results suggest an inability of epididymal adipocytes of NOX2-KO mice to undergo hyperplasia in response to overnutrition. Although NOX2 is expressed in human preadipocytes, its expression levels do not change upon differentiation (41). Preadipocyte expression levels of both NOX1 and NOX4 increase upon insulin-induced differentiation, but it has been shown that NOX4 is in fact the isoform that acts as the switch from proliferation to differentiation in human preadipocytes (41). Based on these findings, it is unlikely that NOX2 plays a role in adipocyte differentiation, and since NOX2 is not expressed in murine adipocytes (21), extra-adipocyte factors are likely responsible for this lipid storage defect.

Our most relevant findings pertain to the status of the liver, as NOX2-KO mice stored far more lipid in liver than WT mice on either Chow or HF diets. The striking increase in hepatic mass contributed to the gain in body weight and tracked with the storage of the excess energy consumed by the NOX2-KO compared with WT mice. NOX2-KO mice had lower expression of genes involved in de novo lipogenesis compared with WT mice, suggesting that NOX2-KO livers responded appropriately to increased accumulation of lipid. It is likely that the defect leading to hepatic steatosis in NOX2-KO mice is upstream of the liver (e.g., at the level of the hypothalamus). In addition to advanced liver steatosis, NOX2-KO mice had higher macrophage markers and higher indexes of hepatic inflammation. These cells may be resident Kupffer cells and/or monocytes/macrophages recruited from the circulation. The exacerbated inflammatory state of the liver likely contributes to the insulin resistance observed in the HF-fed NOX2-KO mice. The impact of NOX2 gene deletion on inflammation is variable, depending on the endpoint assessed. Consistent with our finding, Lee et al. (30) found increased inflammation and tissue infiltration of immune cells in NOX2-KO mice compared with WT mice during the development of arthritis. Moreover, Pepping et al. found increased macrophage infiltration into EWAT of HF-fed WT mice, but not NOX2-KO mice (38), whereas we found a trend for increased EWAT macrophage infiltration in both Chow- and HF-fed NOX2-KO mice compared with WT mice. On the other hand, You et al. observed decreased kidney macrophage infiltration in NOX2-KO mice in a model of type
1 diabetes (50) attributed to lower levels of kidney Cel2 (monocyte chemoattractant protein-1/MCP-1). Hence, changes in immune cell infiltration of tissues occur in NOX2-KO mice, but these vary according to the location and challenges imposed.

Chow-fed NOX2-KO mice showed slightly lower fasting glucose levels throughout the study as well as during the glucose tolerance test. A previous study by Li et al. (31) on similar NOX2-KO mice also noted mild hypoglycemia during the glucose tolerance test in chow-fed mice (although no difference was noted in fasting glucose). The authors linked this phenotype to the observation that knockdown of NOX2 in pancreatic islets elevated insulin secretion in response to high glucose. Similar to Li et al. (31), we did not observe any differences in fasting insulin in chow-fed NOX2 mice compared with WT mice; however, our HF-fed NOX2-KO mice became hyperinsulinemic after only 7 wk on the diet, whereas HF-fed WT mice still had normal fasting insulin levels. This hyperinsulinemia is consistent with the higher insulin resistance of HF-fed NOX2-KO mice compared with HF-fed WT mice. The advanced progression of insulin resistance in NOX2-KO mice was likely due to pronounced hepatic steatosis and increased liver macrophage infiltration; however, since NOX2-KO mice were hyperphagic and heavier than WT mice, a pair-feeding study will be necessary to separate the effects of NOX2 from overnutrition and body weight on insulin resistance.

In summary, the major phenotypic manifestations of NOX2-KO mice compared with WT mice are their hyperphagia, excessive deposition of lipid in liver, and decreased reliance on EWAT for lipid storage. Future studies will be required to dissect whether one or more cell types trigger this phenotype, but we can speculate on some possibilities. The origin of the hyperphagia is possibly complex, and may include a failure to increase hypothalamic Pmc expression in response to overnutrition as well as responses to alterations in adipose and hepatic factors. Given that NOX2 is not expressed in murine adipocytes, the reduced gain in EWAT mass and preferential lipid partitioning to the liver displayed by NOX2-KO mice is likely due to extra-adipocyte factors. Regardless of the cellular origin(s) of the phenotype, NOX2-KO mice reveal that NOX2 is required for proper metabolic appetite control and prevention of hepatic steatosis. While establishing the cellular basis for each of these defects is necessary and should be undertaken through cell type-specific NOX2 deletion approaches, the current model has already shed important information on the impact of NOX2 on these functions. These studies highlight the importance of an NADPH oxidase that is typical of myeloid cells in the proper control of food intake and shuttling of dietary lipid. Moreover, our study suggests that inhibiting NOX2 for therapeautic purposes may reduce the inflammatory tone of macrophages but may divert dietary fats to the liver, promoting steatosis, inflammation, and whole body insulin resistance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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