Phenotypic abnormalities in macrophages from leptin-deficient, obese mice

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Lee, Fung-Yee J anet, Yunbo Li, Eun K. Yang, Shi Qi Yang, Hui Zhi Lin, Michael A. Trush, Andrew J. Dannenberg, and Anna Mae Diehl. Phenotypic abnormalities in macrophages from leptin-deficient, obese mice. Am. J. Physiol. 276 (Cell Physiol. 45): C386–C394, 1999.—Obesity is a complex syndrome that involves defective signaling by a number of different factors that regulate appetite and energy homeostasis. Treatment with exogenous leptin reverses hyperphagia and obesity in ob/ob mice, which have a mutation that causes leptin deficiency, proving the importance of this factor and its receptors in the obesity syndrome. Cells with leptin receptors have been identified outside of the appetite regulatory centers in the brain. Thus leptin has peripheral targets. Because macrophages express signaling-competent leptin receptors, these cells may be altered during chronic leptin deficiency. Consistent with this concept, the present study identifies several phenotypic abnormalities in macrophages from ob/ob mice, including decreased steady-state levels of uncoupling protein-2 mRNA, increased mitochondrial production of superoxide and hydrogen peroxide, constitutive activation of CCAAT enhancer binding protein (C/EBP)-β, an oxidant-sensitive transcription factor, increased expression of interleukin-6 and cyclooxygenase (COX)-2, two C/EBP-β target genes, and increased COX-2-dependent production of PGE_2. Given the importance of macrophages in the general regulation of inflammation and immunity, these alterations in macrophage function may contribute to obesity-related pathophysiology.

Obesity; uncoupling proteins; cytokines; cyclooxygenase-2; superoxide

Obesity is a major health problem in affluent societies. It is estimated that 30% of the current population in the United States are obese and that the incidence is increasing (19). Epidemiologic studies have demonstrated that obesity causes premature mortality in adults (38). This finding is consistent with evidence that obesity promotes a wide array of diseases, including cardiovascular disease, breast and colorectal cancer, osteoarthritis, diabetes, and cirrhosis (4, 23, 26, 29). Superficially, the treatment for obesity-related medical problems seems simple, i.e., weight reduction. However, once obesity has developed, efforts to return to normal body mass are typically unsuccessful and may actually increase mortality (1, 16, 29). Although much research has been devoted to understanding the mechanisms that drive hyperphagia and adiposity (reviewed in Ref. 34), very little work has focused on other end-organ consequences of obesity. However, efforts to delineate the mechanisms responsible for obesity-related organ damage are important because the latter increase morbidity and mortality in obese individuals.

Study of rodents with heritable obesity indicates that obesity is a complex syndrome that involves defective signaling by a number of different factors that regulate appetite and energy homeostasis (3, 10, 27, 34, 37). The hormone leptin and its receptors are important components of this system. Leptin is produced predominantly by adipocytes and acts on specific neuronal targets in the ventral median nucleus of the hypothalamus to inhibit appetite. Mice with homoygous loss-of-function mutations in the obese (ob) gene, which encodes leptin, or in the diabetes (db) gene, which encodes the leptin receptor, are hyperphagic and become obese (5). Both strains of mice are also insulin-resistant, hyperlipemic, and relatively infertile, and many of these problems are corrected by treatment with exogenous leptin (30). The fact that such a complex phenotype results from absolute or relative leptin deficiency suggests that leptin may have many targets. Consistent with this possibility is emerging evidence that many cells express either short (extracellular domains) or long (membrane spanning) forms of the leptin receptor (12, 39, 44). Short forms of the leptin receptor do not appear to transduce intracellular signals and probably provide a tissue reservoir of leptin. Long forms of the leptin receptor have homology to glycoprotein-130 (39) and activate many of the same intracellular signaling pathways that are initiated by interleukin-6 (IL-6), including induction of Janus kinases and signal-transducing activators of transcription (13).

Macrophages express transmembrane forms of the leptin receptor, and recombinant leptin has been shown to increase phagocytic activity and macrophage monocyte colony-stimulating factor release by peritoneal macrophages from normal mice (12, 25). Previously, we reported that phagocytic activity is decreased in macrophages harvested from leptin-deficient ob/ob mice and leptin-resistant db/db mice and demonstrated that recombinant leptin normalizes phagocytosis by cultured ob/ob macrophages but not db/db macrophages (which lack functional leptin receptors) (24). These results indicate that leptin interacts with its receptors to regulate important macrophage functions and suggest that chronic leptin deficiency may alter the phenotype of macrophages. If so, then macrophage dysfunction may be a primary abnormality in obesity syndromes.
that result from leptin insufficiency or leptin resistance. Furthermore, defective macrophages could play a role in many obesity-related diseases, since these cells are normally important regulators of inflammation and immunity.

Because leptin deficiency affects multiple hormonal and metabolic responses, it is often difficult to determine which, if any, aspects of obesity-related pathophysiology result directly from the interruption of leptin-leptin receptor interactions. Several leptin-regulated genes have been identified in cells (e.g., adipocytes) that express transmembrane leptin receptors. For example, leptin is known to induce the expression of uncoupling protein (UCP)-2 in adipocytes (35). Macrophages also express UCP-2 (12, 20, 28), although it is not clear whether or not leptin regulates UCP-2 expression in these cells. If, however, leptin does directly or indirectly modulate macrophage UCP-2 activity, then leptin deficiency might alter energy homeostasis and oxidant production by these cells, since UCP is known to regulate mitochondrial respiration (11, 17, 28, 33).

The purpose of the present work was to determine whether the chronic leptin-deficient state alters the phenotype of macrophages and to evaluate the role of UCP-2, a leptin-regulated gene product, in this process. Our results demonstrate that UCP-2 expression is suppressed in macrophages from leptin-deficient mice and suggest that this is associated with increased mitochondrial oxidant generation, the activation of oxidant-sensitive transcription factors, and the induction of some genes that are transactivated by these proteins.

MATERIALS AND METHODS

Animals. The ob/ob mice and their lean (+/ob) littersmates were purchased from Jackson Laboratories. Animals were maintained in a temperature-controlled environment with a 12:12-h light-dark cycle and fed standard pellet chow diet. All animal experiments were done in accordance with National Institutes of Health and Johns Hopkins University guidelines for the humane use of laboratory animals.

Reagents. All chemicals were purchased from Sigma, with the following exceptions. Bicinchoninic acid (BCA) kits for protein assays came from Pierce. Sodium isothiocyanate, Taq polymerase, RT, and proteinase inhibitor solution were purchased from Boehringer Mannheim (Indianapolis, IN). Nylon membranes were purchased from DuPont (Boston, MA). Medium (DMEM), bovine serum, and plastic dishes for the cell culture experiments came from Gibco. Enzyme immunoassay reagents for PGE2 assays were from Cayman (Ann Arbor, MI). The oligomer containing the CCAAT enhancer binding protein (C/EBP) binding site in the 422 promoter and the C/EBP binding protein (C/EBP) were gifts from M. D. Lane (Johns Hopkins University, Baltimore, MD). Dr. Gennaro Ciliberto (Research Institute, Rome, Italy) provided the cDNA for IL-6. The cyclooxygenase (COX)-2 cDNA was given to us by Dr. Raymond DuBois (Vanderbilt University, Nashville, TN).

Peritoneal macrophage experiments. Mice were injected with 3 cm3 of 3% thioglycollate intraperitoneally. Five days later, animals were euthanized by CO2 inhalation, and the peritoneal cavity was immediately lavaged with Hanks’ balanced salt solution with EDTA (pH 7.2) to harvest the thioglycollate-elicted macrophages. Macrophages from ~10 mice/group were pooled and plated on plastic culture dishes (105 cells/cm2) and cultured overnight in DMEM with 10% bovine serum (in studies to evaluate nuclear proteins, RNA, or mitochondrial oxidant generation) or DMEM without serum (to evaluate PGE2 production). Experiments were done the following morning. All experiments were repeated at least twice. Each experiment studied macrophages pooled from 10 ob/ob mice or from 10 control mice. Thus the final results were obtained from three different sets of macrophages pooled from a total of 30 ob/ob and 30 control mice.

RNA isolation and analysis. Vehicile or lipopolysaccharide (LPS; from Escherichia coli serotype 0111:B4; 1 µg/ml) was added to macrophage cultures, and 90 min later cells were harvested. Total RNA was isolated from the fresh cell pellets according to the method of Chomczynski and Sacchi (7), as we have described (41). RNA was quantitated by measuring its absorbance at 260/280 nm, and its quality was assessed by electrophoresis on ethidium bromide-stained agarose gels under denaturing conditions. To evaluate potential differences in the gene expression of ob/ob and lean macrophages, total RNA (20 µg/lane) was separated by agarose gel electrophoresis under denaturing conditions and transferred to nitrocellulose membranes by capillary transfer and then were rinsed with 1% methylene blue and photographed to document lane-to-lane variations in RNA. Membranes were hybridized overnight at 42°C with 32P-labeled cDNAs for UCP-2, IL-6, COX-2, or 18S. After a washing under stringent conditions, membranes were exposed to X-ray film. IL-6 gene expression was also evaluated by semi-quantitative RT-PCR as described (31, 32). Briefly, total liver RNA (3 µg) was reverse transcribed and amplified with specific internal oligonucleotide primers in a thermocycler under semi-quantitative conditions. The expression of glyceraldehyde-3-phosphate dehydrogenase, a constitutively expressed gene, was evaluated in parallel assays. PCR products were separated by agarose gel electrophoresis. After transfer to nylon membranes by capillary blotting and hybridization with IL-6-specific probes, the products were visualized by enhanced chemiluminescence. All RNA experiments, using pooled macrophages from 10 mice per group per experiment, were repeated 3 times to assure reproducibility of results.

Protein nuclear isolation and gel mobility shift assays. Macrophages were harvested and cultured as described above, except that cultures were harvested at several different time points (0, 0.5, 1, or 1.5 h) after vehicle or LPS treatment. Nuclear proteins were isolated according to the method of Lavery and Schibler (21), as we have described (42). Briefly, cells were homogenized in homogenizing buffer (10 mM Tris·HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, and 1.4 mM spermidine, 0.03 mM spermine, 1 EDTA, 0.5 mM EGTA, and 1.4 mM MgCl2) and nuclear proteins were isolated according to the method of Chomczynski and Sacchi (7), as we have described (42). Nuclear proteins were isolated according to the method of Lavery and Schibler (21), as we have described (42). Formaldehyde-treated DNA samples (10 µg) were added to nuclear protein samples (5 µg), the mixture was incubated for 10 min at room temperature with 30 µl of 3% formaldehyde, and DNA was cross-linked by UV illumination. Nuclear proteins were washed, and DNA was eluted from the DNA-protein complexes with sodium citrate and salt solution (0.1 M NaCl, 0.01 M EDTA) before electrophoresis on 5% polyacrylamide-7 M urea gels. DNA-protein complexes were visualized with ethidium bromide staining, and digital photographs were obtained.

Gel mobility supershift assays. DNA binding activity of C/EBP (isotypes C/EBPα, C/EBPβ, and C/EBPδ) was determined by adding specific antibodies (anti-C/EBPα, anti-C/EBPβ, and anti-C/EBPδ) to the EMSA reaction mixtures. Complexes were visualized by ethidium bromide staining, and digital photographs were obtained.
stranded oligonucleotide probes. Reaction mixtures were loaded onto 5% polyacrylamide gels and separated by electrophoresis under nondenaturing conditions. After drying, gels were exposed to X-ray film to demonstrate protein-probe complexes. In some reactions, preimmune sera or antisera (1 µl/lane) to specific DNA binding proteins were added to the reaction mixtures during the incubation. The specificity of the protein-probe complexes revealed on subsequent electrophoresis was assured if the addition of the preimmune antisera had no effect on complex formation but addition of the specific antisera either disrupted complex formation with the probe or produced a “supershift” of the protein-probe complex (i.e., retarded complex mobility) on the gel. Differences in the intensity of transcription factor binding activity were evaluated by phosphorimager analysis of the dried gels.

**Results**

The yield and viability of macrophages harvested from the peritoneal cavities of obese (ob/ob) mice and their lean (/?/ob) littermates were similar. However, closer inspection of macrophages pooled from obese mice and those pooled from lean mice revealed several phenotypic differences. As shown in Fig. 1, UCP-2 was expressed in cultured peritoneal macrophages from lean mice, and a 90-min exposure of these cultures to LPS decreased UCP-2 expression by ~40%. The ob/ob macrophages expressed only about one-half as much UCP-2 as the lean macrophages basally. These low basal levels of UCP-2 transcripts were not further reduced following LPS treatment.

UCP-2 activity is thought to uncouple oxidative phosphorylation and decrease O2 generation by normal peritoneal macrophages (28). To determine whether differences in UCP-2 expression in lean and ob/ob macrophages were associated with differences in mitochondrial oxidant generation, cell suspensions were incubated with either lucigenin or luminol plus HRP to quantitate mitochondrion-derived O2 and H2O2 generation, respectively. The ob/ob macrophages produced more O2 (Fig. 2, A and C) and H2O2 (Fig. 2B) than the lean macrophages basally. LPS exposure significantly increased oxidant production by lean macrophages but had less of an effect in ob/ob cells, which were already

![Fig. 1. Uncoupling protein (UCP)-2 mRNA expression in macrophages from ob/ob mice and their lean littersmates. Thioglycollate-elicited peritoneal macrophages were isolated from ob/ob mice and their lean littersmates on 3 different occasions. In each experiment, macrophages were pooled from 10 lean and 10 ob/ob mice before cells from each group were plated onto plastic dishes (106 cells/dish). After overnight culture in serum-containing medium, lipopolysaccharide (LPS; 1 µg/ml) or vehicle was added to the cell suspensions (0.5-1 × 106 cells/ml), and lucigenin- or luminol-derived chemiluminescence was measured in a six-chamber Berthold LB9505 luminometer at 37°C for 40 min, as described (22). Lucigenin-derived chemiluminescence measures intramitochondrial superoxide anion (O2) generation, and luminol-derived chemiluminescence measures extracellular, mitochondrion-derived hydrogen peroxide (H2O2) production. To confirm the importance of mitochondria as the source of these oxidants, inhibitors of mitochondrial respiration, rotenone (10 µM; a complex I inhibitor) and myxothiazol (10 µM; a complex III inhibitor), were added to some assays to determine whether this reduces either O2 or H2O2 generation. ATP production was evaluated according to the manufacturer’s protocols with commercially available luciferin-luciferase ATP assay kits (Sigma), using similar approaches to monitor chemiluminescence. Briefly, to each well, 50 µl complete PBS, 50 µl H2O, and 100 µl ATP-releasing reagent were added to lyse cells. Two minutes later, 100 µl of the cell lysate were added into a cuvette containing 100 µl ATP assay mix. The luminescence was determined immediately on a luminometer (Berthold, LB9505). ATP content was calculated using a concurrently run standard curve and expressed as percentage of untreated control cells.**
producing high levels of $O_2$ and $H_2O_2$. Thus there appeared to be an inverse correlation between oxidant production and UCP-2 mRNA levels. Basal expression of UCP-2 was high in lean macrophages, and these cells produced low levels of $O_2$ and $H_2O_2$ basally. In contrast, ob/ob cells expressed much less UCP-2 and produced much more $O_2$ and $H_2O_2$ basally. In lean macrophages, LPS stimulation resulted in a significant fall in UCP-2 mRNA and led to a marked increase in oxidant generation. LPS had less of an effect on the already low levels of UCP-2 in ob/ob macrophage, and LPS treatment was also followed by a smaller increase in the already high rates of oxidant production by these cells.

Results displayed in Fig. 3 support the concept that mitochondria are the major source of the oxidants that were measured during the previous experiments. Addition of electron transport chain inhibitors that block respiration at complex I (rotenone) and complex III (myxothiazol) virtually eliminates $O_2$ generation in both ob/ob and lean macrophages (Fig. 3A). Furthermore, treatment of ob/ob cells with carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone (FCCP), a known mitochondrial uncoupling agent, significantly reduces their $O_2$ generation, despite continued low expression of endogenous UCP-2 (Fig. 3B). The latter finding suggests that decreased UCP-2 may contribute to the dysregulation of oxidant production by ob/ob macrophages.

Mitochondrial uncouplers are also thought to influence ATP production. Uncoupling of mitochondrial electron transport from ATP synthesis is predicted to decrease cellular ATP stores (17). Commercially available luciferase assays were used to evaluate ATP production in untreated macrophage suspensions. As predicted, ob/ob macrophages, which express less UCP-2 (Fig. 1) and which generate more mitochondrion-derived oxidants (Fig. 2), also have greater ATP concentrations than lean macrophages (Fig. 4).

Cellular oxidants are known to influence the redox state of oxidant-sensitive transcription factors, and this may influence the DNA binding activity of these proteins (6, 18). Because the degree of mitochondrial oxidants produced by ob/ob and lean macrophages differs, it is conceivable that these cells may also exhibit differences in the DNA binding activities of oxidant-sensitive DNA binding proteins, such as C/EBP-β (18). To evaluate this possibility, differences in the DNA binding activity of this oxidant-sensitive...
transcription factor were evaluated by gel mobility shift assays (Fig. 5). The DNA binding activity of C/EBP-β is increased transiently by LPS treatment in lean macrophages. However, C/EBP-β binding activity is apparent in ob/ob macrophages even before LPS exposure. Thus, compared with lean macrophages, ob/ob macrophages, which produce more O2 and H2O2 basally (Fig. 2), also have more constitutive C/EBP-β binding activity. LPS induces C/EBP-DNA binding activity transiently in lean macrophages, and, as noted for UCP-2 and mitochondrial oxidant production, LPS had less of an effect in ob/ob macrophages. However, C/EBP-DNA binding activity remained generally greater in ob/ob macrophages than in lean macrophages after LPS treatment.

Several genes that are induced during inflammation are transcriptionally regulated by C/EBP-β (2, 40). For example, the transcription of COX-2, which encodes an enzyme that metabolizes arachidonic acid to prostanooids, is activated by this DNA binding protein (8, 14, 36). Because ob/ob and lean macrophages exhibit differences in the DNA binding activities of C/EBP-β, it is possible that these cells may have different levels of COX-2, either basally or following treatment with LPS, a potent inducer of COX-2. To evaluate this possibility,
Northern blot analysis was used to compare steady-state levels of COX-2 mRNA in the two groups of cells. As shown in Fig. 6, before LPS treatment, COX-2 mRNAs could not be detected by Northern blot analysis in either group. LPS treatment substantially induced COX-2 in both groups, but COX-2 expression was about fivefold greater in ob/ob than in lean cells following LPS treatment.

COX-2 catalyzes the synthesis of PGE₂ from arachidonate. Hence, during conditions in which substrate (arachidonate) is not limiting, PGE₂ production is a good measure of COX-2 activity (8, 14, 36). To determine whether COX-2 activity is different in ob/ob and lean macrophages, PGE₂ concentrations were evaluated in macrophage-conditioned medium harvested from cells that were cultured in medium alone or medium plus sodium arachidonate. Compared with macrophages from lean mice, macrophages from ob/ob mice produce more PGE₂ both basally and when incubated with sodium arachidonate (Table 1). Thus COX-2 activity is generally greater in ob/ob macrophages than in macrophages from their lean littermates.

PGE₂ has many effects, including the induction of IL-6 (15). Thus it is possible that ob/ob macrophages, which produce more PGE₂, also produce more IL-6. To evaluate this possibility, IL-6 expression in ob/ob and lean macrophages was evaluated by Northern blot analysis and RT-PCR assay. As shown in Fig. 7A, IL-6 mRNA is not detected by Northern blot analysis in either ob/ob or lean macrophages before LPS treatment. LPS induces IL-6 in both groups, but the degree of induction is much greater in ob/ob macrophages than in macrophages from lean controls. RT-PCR analysis is a more sensitive technique than Northern blot analysis for demonstrating low-abundance mRNAs. When this approach was used, basal differences in IL-6 expression were identified between ob/ob and lean macrophages. IL-6 transcripts were barely detected by RT-PCR assay of total RNA from lean macrophages but were easily identified in ob/ob macrophages (Fig. 7B). Thus ob/ob macrophages produce more PGE₂ and more IL-6 than lean macrophages, both before and after LPS.

### DISCUSSION

Obesity is a complex syndrome that results from an imbalance in energy intake and utilization. Several different factors interact to regulate appetite by influencing the activity of neuronal melanocortin type 4 receptors in the ventral median nucleus of the hypothalamus (27). Many of these factors, including the appetite-suppressing hormone leptin, also trigger effects in peripheral tissues. Some of the latter appear to result from direct interactions between appetite-regulating factors and their receptors on cells that reside outside the central nervous system (5). It is likely that these peripheral effects contribute to defective energy utilization and some of the end-organ damage that occurs in obese individuals.

Results of the present study identify several phenotypic abnormalities in macrophages of chronically lep-
Fig. 7. Interleukin-6 (IL-6) expression in macrophages from ob/ob mice and their lean littermates. On 3 occasions, total RNA was isolated from pooled, thioglycollate-elicited peritoneal macrophages as described in MATERIALS AND METHODS and analyzed as detailed for Figs. 1 and 6. A: Northern blot analysis of IL-6 expression. Total RNA (20 µg/lane) was separated by electrophoresis on agarose gels under denaturing conditions, transferred to nylon membranes by capillary diffusion, and hybridized with labeled IL-6 cDNAs. After a washing under stringent conditions, membranes were exposed to X-ray film. Top: representative Northern blot showing IL-6 mRNAs from lean and ob/ob cells before (-) and after (+) 90-min exposure to LPS, together with 18S RNA expression on same membrane. Bottom: means ± SD from 3 Northern blots (1 from each experiment). B: RT-PCR analysis of IL-6 expression in untreated macrophages. Total RNA from untreated lean and ob/ob macrophages that was used for Northern blot studies was also evaluated by RT-PCR; 3 µg total RNA was reverse transcribed and amplified in a thermocycler under semi-quantitative conditions with internal oligonucleotide primers specific for murine IL-6 as described in METHODS. Top: representative Southern blot of basal IL-6 expression in 2 groups, together with GAPDH expression in each of these samples. Bottom: means ± SD from 3 experiments, each of which used input RNA from macrophages pooled from different ob/ob and lean mice.
concept is supported by observations that ob/ob macrophages exhibit higher constitutive DNA binding activity of the LPS-regulated transcription factor C/EBP-β and increased expression and/or activity of some of its target genes, such as those for COX-2 and IL-6. Thus macrophages from chronically leptin-deficient animals appear to be constitutively activated, such that they overproduce a variety of inflammatory mediators, including oxidants, cytokines, and prostanooids.

Although macrophages are critical cells in the immune response and in host defense mechanisms, they have also been implicated as mediators of a number of pathologies. Thus it is possible that these abnormalities in macrophage phenotype may contribute to some of the end-organ complications of obesity that have been observed in ob/ob mice. Circulating leptin levels are generally increased in human obesity and in several other genetically obese strains of mice and rats (e.g., with the diabetes, fatty, agouti, or tubby mutations) (34). Presumably, in the latter situations, obesity results, at least in part, from resistance to leptin-initiated signals in the hypothalamus, because hyperphagia occurs despite increased levels of a potent anorectic factor. Additional work will be required to determine whether macrophage dysfunction is a general feature of the obese phenotype and whether peripheral leptin resistance or other, as yet unrecognized, hormonal disturbances are responsible for the deficits. This effort appears well justified, however, since macrophages regulate inflammation and immunity and thus may participate in the pathogenesis of several diseases that contribute to obesity-related morbidity and mortality.

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REFERENCES


