Increased pulmonary responses to acute ozone exposure in obese db/db mice

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Lu, Frank L., Richard A. Johnston, Lesley Flynt, Todd A. Theman, Raya D. Terry, Igor N. Schwartzman, Anna Lee, and Stephanie A. Shore. Increased pulmonary responses to acute ozone exposure in obese db/db mice. Am J Physiol Lung Cell Mol Physiol 290: L856–L865, 2006.—Epidemiological studies indicate that the prevalence of asthma is increased in obese and overweight humans. Responses to ozone (O3), an asthma trigger, are increased in obese (ob/ob) mice lacking the satiety hormone leptin. The long form of leptin receptor (Ob-Rb) is required for satiety; mice lacking this receptor (db/db mice) are also substantially obese. Here, wild-type (WT) and db/db mice were exposed to air or O3 (2 ppm) for 3 h. Airway responsiveness, measured by the forced oscillation technique, was greater in db/db than WT mice after air exposure. O3-induced increases in pulmonary resistance and airway responsiveness were also greater in db/db mice. BALF eotaxin, IL-6, KC, and MIP-2 increased 4 h after O3 exposure and subsided by 24 h, whereas protein and neutrophils continued to increase through 24 h. For each outcome, the effect of O3 was significantly greater in db/db than WT mice. Previously published results obtained in ob/ob mice were similar except for O3-induced neutrophils and MIP-2, which were not different from WT mice. Ob/ob also induced pulmonary IL-1β and TNF-α mRNA expression in db/db but not ob/ob mice. Leptin was increased in serum of db/db mice, and pulmonary mRNA expression of short form of leptin receptor (Ob-Ra) was similar in db/db and WT mice. These data confirm obese mice have innate airway hyperresponsiveness and increased pulmonary responses to O3. Differences between ob/ob mice, which lack leptin, and db/db mice, which lack Ob-Rb, but not Ob-Ra, suggest leptin, acting through Ob-Ra, can modify some pulmonary responses to O3.

leptin; interleukin-1β; airway responsiveness; macrophage inflammatory protein-2; neutrophil; ventilation

EPIDEMIOLOGICAL DATA INDICATE that the prevalence of asthma is increased in the obese (21). Studies using objective measures of asthma, such as bronchodilator response, peak flow variability, or airway responsiveness, have confirmed this association (12, 13, 15, 56). It is likely that obesity either causes or worsens asthma. Longitudinal studies indicate that obesity antedates asthma and that the relative risk of incident asthma increases with increasing body mass index (11, 12, 43). Furthermore, morbidly obese asthmatics studied after weight loss exhibit decreased severity and symptoms of asthma (1, 24, 53). Obesity may be particularly important for severe asthma since >75% of those visiting the emergency room for asthma are obese or overweight (56). In addition, ~70% of severe asthmatics are obese (2), and in women, asthma severity increases with body mass index (59).

We have been investigating animal models that can be used to examine the mechanistic basis for the relationship between obesity and asthma. Ob/ob mice are genetically deficient in leptin, a satiety hormone, and Cpefat mice are genetically deficient in carboxypeptidase E, an enzyme involved in processing of neuropeptides involved in satiety. Both ob/ob and Cpefat mice are obese, and both types of mice exhibit innate airway hyperresponsiveness (AHR), a characteristic feature of asthma (36, 46, 50). In addition, in both ob/ob and Cpefat mice, airway responses to ozone (O3), a common asthma trigger, are augmented (36, 50). Together, these results suggest that the effects of obesity on airway function are independent of the modality of obesity. However, differences exist between ob/ob and Cpefat mice with respect to their airway responses to O3. For example, O3-induced increases in bronchoalveolar lavage (BAL) neutrophils are greater in Cpefat than in wild-type mice (36), whereas this is not the case in ob/ob mice even though certain BAL inflammatory cytokines and chemokines are elevated in ob/ob vs. wild-type mice (50). These differences may be related to the satiety hormone leptin, which is proinflammatory (32). Ob/ob mice are genetically deficient in leptin (38), whereas Cpefat mice have elevated serum leptin levels (36), similar to obese humans.

Leptin suppresses appetite and increases metabolism by binding to the long isoform of the leptin receptor (Ob-Rb) in the hypothalamus. This results in signal transducer and activator of transcription (STAT)-3 activation, an event required for leptin’s effect on satiety and metabolism (23, 55). In the db/db mouse, the cytoplasmic domain of Ob-Rb is truncated due to a genetic mutation (14), and leptin-induced STAT-3 activation is lost (23). For the most part, the phenotype of the db/db mouse is similar to that of the ob/ob mouse: hyperphagia, hypometabolism, hyperinsulinemia, and obesity (38). Nevertheless, many differences between db/db and ob/ob mice have been reported (17, 20, 33, 47). For example, ob/ob mice are hypersensitive to the anorectic effects of lipopolysaccharide, whereas db/db mice are resistant (20). Dbl/db mice develop mesangial expansion similar to that of human diabetic nephropathy, whereas ob/ob mice do not (17). These differences likely result from leptin signaling through leptin receptor isoforms other than Ob-Rb in the db/db mouse. Several leptin receptor isoforms are generated by alternative splicing of the gene (41), and whereas the db/db mouse lacks Ob-Rb, other isoforms are expressed (23, 27). These short forms of the leptin...
receptor have truncated (Ob-Rα, Ob-Rc, Ob-Rd) or absent (Ob-Rx) cytoplasmic domains. These leptin receptors are expressed in peripheral tissues, especially in lung tissue, which expresses Ob-Rx at higher levels than any other tissue (41). These short isoforms lack the ability to induce STAT-3 activation but are capable of some types of signaling, including JAK, ERK, and phosphatidylinositol 3-kinase activation (7, 37), and leptin can cause changes in cell function even in cells that do not express Ob-Rα (27, 47). The functional significance of leptin signaling through these receptors for lung function remains to be established, but may be important, since leptin is proinflammatory (32) and has been shown to augment airway responsiveness to both O3 (50) and allergen (52). For this reason, we examined airway responsiveness and airway inflammation in wild-type (C57BL/6) and db/db mice following acute (3-h) O3 exposure. Our results indicate that whereas db/db mice exhibit an airway phenotype similar to ob/ob mice in many respects, there are also several notable differences between these mice, which may be due to leptin signaling through receptor isoforms other than Ob-Rα.

MATERIALS AND METHODS

Animals. All experimental protocols used in this study were approved by The Harvard Medical Area Standing Committee on Animals. Female db/db and ob/ob mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used between 8 and 12 wk of age. Age- and gender-matched wild-type controls were purchased at the same time. The db/db and ob/ob mice used in this study were on a C57BL/6J background; thus wild-type C57BL/6J mice were used as controls. All mice were fed standard mouse chow (Pico Mouse Diet 5058, Pharmaserve).

Protocol. Db/db and wild-type mice were exposed to O3 (2 ppm) or to room air for 3 h. Twenty-four hours later, mice were anesthetized and instrumented for the measurement of pulmonary mechanics by the forced oscillation technique, and airway responsiveness to intravenous (iv) methacholine (MCh) or serotonin was measured. We chose to examine airway responsiveness 24 h after the cessation of O3 exposure based on previous reports using this species (62). Once these measurements were completed, the mice were killed with pentobarbital sodium (50 mg/kg). The trachea was cannulated with a tubing adaptor, and the tail vein was cannulated for the delivery of acetyl-β-methylcholine chloride (MCh; Sigma-Aldrich, St. Louis, MO) or serotonin (5-HT; Sigma-Aldrich). A wide incision in the chest wall was made bilaterally to expose the lungs to atmospheric pressure and to exclude any chest wall contribution to pulmonary mechanics. The mice were then ventilated at a VT of 0.3 ml using a specialized ventilator (flexiVent; SCIREQ, Montreal, Canada). Frequency was set at 150 and 180 Hz in wild-type and db/db mice, respectively. The slightly higher frequency used for the db/db mice was chosen to conform to their spontaneous breathing frequencies (vive infra), whereas during spontaneous breathing, Vt did not differ between the strains. A positive end-expiratory pressure (PEEP) of 3 cmH2O was applied by placing the expiratory line under water. Baseline pulmonary mechanics and responses to iv MCh or 5-HT were measured using the forced oscillation technique, as described previously (50). To obtain dose-response curves to iv MCh, mice were given an inflation to three times Vt. One minute later, PBS was administered (1 μl/g), and total lung resistance (Rl) was measured using a 2.5 Hz sinusoidal forcing function every eighth breath for the next 1–2 min, until Rl peaked and began to decline. The mouse was then given another inflation to three times Vt. The procedure was repeated using doses of MCh dissolved in PBS increasing in approximate half-log intervals from 0.03–3.0 mg/ml at a dose of 1 μl/g. The five highest values of Rl obtained following each dose were averaged to obtain the final values for each dose. 5-HT dose-response curves were obtained in an identical manner except that we used concentrations of 5-HT of 0.01–0.3 mg/ml. MCh and 5-HT dose-response curves were obtained in different cohorts of mice.

BAL. BAL was performed as described previously (50). The supernatant was collected and stored at −80°C. Total BALF cells and differentials were determined as described previously (50). The total BALF protein concentration was determined spectrophotometrically according to the Bradford protein assay procedure (Bio-Rad, Hercules, CA). The concentrations of BALF or serum adiponectin, eotaxin, IL-6, KC, leptin, macrophage inflammatory protein-2 (MIP-2), monocyte chemotactic protein-1 (MCP-1), sTNFR1, and sTNFR2 were determined with either ELISA or DuoSet ELISA kits (Panomics, Redwood City, CA for adiponectin; and R&D Systems, Minneapolis, MN for all others) according to the manufacturers’ instructions.

Histological examination of lung tissue. After BAL, the lungs of air-exposed wild-type and db/db mice were fixed in situ with 10% formalin at a pressure of 23 cmH2O. The lungs were then embedded with paraffin. Sections were prepared and subsequently stained with hematoxylin and eosin. Afterward, the sections were examined under a light microscope to determine the inflammation score, a product of the severity and prevalence of inflammation (25). Severity was assigned a numerical value based on the number of inflammatory cell infiltrate layers around the airways and blood vessels (0, no cells; 1, ≤3 cell layers; 2, 4–9 cell layers; 3, ≥10). The prevalence of inflammation was assigned a numerical value according to the percentage of airways and blood vessels in each section encompassed by inflammatory cells (0, no airways; 1, <25%; 2, 25–50%; 3, >50%).

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PEEP of 3 cmH₂O was imposed. Lungs were inflated to three times
V₉ to standardize volume history. For quasistatic PV relationships,
increments in volume, ~0.11 ml, were introduced from end-expiratory
volume using the flexiVent. Airway opening pressure was measured
after each increment in volume was held for 1 s. A second
volume history maneuver was then applied, and 1 min later the
tracheal cannula was clamped at the end of expiration (defined as
open-chested FRC). The lungs were immediately excised, and lung
volume was measured by volume displacement in water. Quasistatic
lung elastance was measured over the deflation portion of the curve
from FRC to FRC plus 0.33 ml.

**RNA extraction and real-time PCR.** Lungs were homogenized
using the PowerGen 125 (Fisher Scientific International, Hampton,
NH) at full speed in 2–3 ml of TRIzol reagent (Invitrogen, Carlsbad,
CA) for 3 min, and total RNA was then extracted in accordance with
the manufacturer’s instructions and stored at −80°C. After RNA
extraction, samples were run through RNeasy RNA Cleanup (QIA-
GEN, Valencia, CA) to increase RNA purity. RT was performed as
previously described (35), and the RT reaction products were stored at
−80°C. Quantitative real-time RT-PCR was performed using an
iCycler iQ Real Time Detection System and iQ SYBR Green Super-
mix in accordance with the manufacturer’s instructions (Bio-Rad).

Primer sets and product sizes for β-actin were as previously described
(35). Primer sets and product sizes for other genes were as follows:
IL-1β forward, 5′-CTG TGT TCC CTG GGA CC-3′; IL-1β
reverse, 5′-CAG CTC ATA TGG GCA CA-3′ (200 bp); Ob-Rα
forward, 5′-ACA CTG TTA ATT TCA CAC A-3′; Ob-Rα
reverse, 5′-AGA TCT GTA AGT ACT GTG CCA T-3′; TNF-α
forward, 5′-GGG ACA GTG ACC TGG ACT GT-3′ (168 bp); TNF-α
reverse, 5′-CTC CCT TTG CAG AAC TCA GG-3′ (111 bp). For
Ob-Rα, a positive control used to construct standard curves for
real-time RT-PCR was generated by cloning the PCR product of
cDNA from the brain of a wild-type mouse into TOPO-TA vectors
(Qiagen). For each set of primers, melting curve analysis yielded a
single peak consistent with one PCR product. Lung mRNA transcript

copy number was assessed relative to β-actin transcript copy number.

**Statistical analysis.** Comparisons of baseline R₉ and BALF parameters
were assessed using factorial ANOVA, using genotype and
exposure as the main effects. Fisher’s least significant differences test
was used as a follow up to determine the significance of differences
between individual groups. Changes in R₉ during MCh and 5-HT
administration and changes in ventilation during O₃ exposure were
assessed by repeated-measures ANOVA. Comparisons of serum
markers of inflammation, displacement lung volume at FRC, quasi-
static elastance, and the pattern of breathing were made by unpaired
Student’s t-tests. STATISTICA software (StatSoft, Tulsa, OK) was
used to perform these analyses. The results are expressed as means ± SE, except where noted. A P value <0.05 was considered significant.

**RESULTS**

**Body weight.** Db/db mice weighed more than twice as much
as age-, strain-, and gender-matched wild-type mice (46.3 ±
0.7 g vs. 18.7 ± 0.3 g).

**Pulmonary mechanics and responsiveness to MCh.** After
air exposure, baseline R₉ was slightly greater in db/db than in
wild-type mice (0.86 ± 0.05 vs. 0.72 ± 0.03
cmH₂O·ml⁻¹·s⁻¹, respectively). O₃ exposure caused a significant
increase in R₉ in both wild-type and db/db mice, but the
effect of O₃ was much greater in db/db (1.83 ± 0.21
cmH₂O·ml⁻¹·s⁻¹) than in wild-type mice (0.87 ± 0.06
cmH₂O·ml⁻¹·s⁻¹). Factorial ANOVA indicated significant
effects of genotype (P < 0.001) and O₃ exposure (P < 0.001)
on baseline R₉ as well as a significant interaction between
genotype and exposure (P < 0.001).

Measurements of airway responsiveness to MCh and 5-HT
were made using R₉ as the outcome indicator. After air
exposure, db/db mice were significantly more responsive to
MCh than wild-type mice (Fig. 1A). O₃ exposure increased
airway responsiveness to MCh in both wild-type and db/db
mice, but the effect of O₃ was greater in the db/db than in the
wild-type mice. Similar results were obtained when 5-HT
instead of MCh was used as the bronchoconstricting agonist
(Fig. 1B). For both 5-HT and MCh, repeated-measures
ANOVA indicated significant effects of the dose of agonist
(P < 0.01), genotype (P < 0.05), and O₃ exposure (P < 0.01)
as well as a significant interaction between genotype and O₃
exposure (P < 0.02).

**Lung volumes and quasistatic PV curves.** We and others
have previously reported that lung size is reduced in ob/ob
mice (50, 54). Therefore, we measured open-chested FRC
(end-expiratory volume at a PEEP of 3 cmH₂O) and obtained
PV curves in unexposed wild-type and db/db mice. FRC was
reduced in db/db (0.15 ± 0.01 ml) vs. wild-type mice (0.25 ±
0.01 ml) (P < 0.05). Consistent with their smaller lung size,
lung PV curves were shifted to the right in db/db vs. wild-type

**Fig. 1.** Changes in lung resistance (Rₑ) induced by intravenous methacholine (A) or se-
rotonin (B) in wild-type (C57BL/6) and db/db mice exposed to air or O₃ (2 ppm for 3 h).
Measurements were made 24 h after exposure. Results are means ± SE of data from 6–11
mice in each group.
mice (Fig. 2), and the lungs were stiffer. Quasistatic lung elastance averaged 19.4 ± 0.6 cmH2O/ml in wild-type mice and 26.9 ± 2.4 cmH2O/ml in db/db mice (P < 0.05). Similar changes in the lung PV curve have been reported in ob/ob mice (54).

O3-induced pulmonary injury and inflammation. BAL was performed after room air exposure and 4 or 24 h after cessation of O3 exposure (2 ppm for 3 h) in wild-type and db/db mice. Note that these db/db mice were exposed at the same time and in the same exposure chambers as wild-type and ob/ob mice whose response to O3 was previously reported (50). BALF inflammatory cells and mediators were not significantly different in lean vs. obese mice after air exposure (Fig. 3). Regardless of genotype, O3 exposure caused a significant increase in BAL IL-6, eotaxin, KC, and MIP-2 measured 4 h after cessation of O3 exposure that declined toward baseline by 24 h. BALF protein and the percentage of BALF cells that were neutrophils were increased 4 h after O3 exposure and increased further at 24 h (Fig. 3). There was a significantly greater increase in all BALF inflammatory parameters in db/db vs. wild-type mice. As described previously (50), qualitatively similar results were obtained in ob/ob mice. However, there were two exceptions. O3-induced changes in the neutrophil chemotactic factor, MIP-2, and in the percentage of BALF cells that were neutrophils were not different between wild-

Fig. 2. Quasistatic pressure-volume (PV) curves measured in open-chested wild-type (C57BL/6) and db/db mice. PV curves were initiated from functional residual capacity (defined as lung volume at 3 cmH2O positive end-expiratory pressure), as measured by subsequent volume displacement. Results are means of 8–10 mice in each group.

Fig. 3. Bronchoalveolar lavage (BAL) protein, IL-6, chemokines, and neutrophils in wild-type (open bars) and db/db (hatched bars) mice. Mice were exposed to air or O3 (2 ppm for 3 h), and BAL was performed 4 or 24 h later. Results are means ± SE of data from 6–7 mice in each group. *P < 0.05 compared with wild-type mice in same exposure group. Note that data for the wild-type mice have been previously published (50). Db/db mice were exposed at the same time in the same exposure chambers as those wild-type mice.
type and ob/ob mice (50), whereas they were different between wild-type and db/db mice (Fig. 3). There was no statistically significant effect of O3 exposure or obesity on the total number of cells recovered from BAL.

We also measured sTNFR1 and sTNFR2 in BALF from db/db and wild-type mice (Fig. 4). Because we had not previously reported results for these outcome indicators from ob/ob mice, they are also included in Fig. 4. Compared with wild-type mice, there was a statistically significant increase in sTNFR1 in BALF from db/db but not ob/ob exposed to air, and a similar nonsignificant trend for sTNFR2. sTNFR1 was increased 4 h after O3 exposure and increased further at 24 h (Fig. 4). At both time points, levels were significantly greater in both ob/ob and db/db mice compared with wild-type controls. There was no significant increase in sTNFR2 4 h after cessation of O3 exposure, but levels were increased by 24 h. No significant effect of obesity was observed for BAL sTNFR2.

We also assessed airway inflammation histologically in air-exposed mice to determine whether there was a cellular inflammatory response in the lung associated with obesity per se. Inflammatory scores were assessed using a semiquantitative technique described by Hamada et al. (25). Inflammatory scores of all wild-type and db/db mice exposed to room air (n = 4 in each group) were 0.

**Pulmonary gene expression.** We measured pulmonary mRNA expression for IL-1β and TNF-α (relative to β-actin mRNA expression) because we and others have shown that deficiencies in the signaling pathways for these cytokines reduce O3-induced responses in mice (16, 45, 51). Compared with air exposure, IL-1β mRNA expression was significantly increased 24 h after O3 exposure in db/db mice, but not in wild-type or ob/ob mice. A similar trend was observed at 4 h but did not reach statistical significance (Fig. 5). Compared with air exposure, TNF-α mRNA expression was significantly increased 24 but not 4 h after O3 exposure in wild-type and db/db mice, but not in ob/ob mice (Fig. 5). To confirm the possibility that leptin may be signaling through the Ob-Rb in the lungs of db/db mice, we used real-time RT-PCR to measure the expression of Ob-Ra mRNA in lungs of wild-type and db/db mice. Ob-Ra mRNA was expressed to the same extent in lungs of wild-type and db/db mice.

**Adipokines and markers of systemic inflammation.** The concentrations of several inflammatory proteins are elevated in the serum of obese humans and mice (10, 28, 29, 36). To examine systemic inflammation in wild-type and db/db mice, we measured the serum levels of leptin, adiponectin, IL-6, and MCP-1, as well as the total number of blood leukocytes in air-exposed mice (Table 1). Serum leptin, MCP-1, and IL-6 were higher in db/db than in wild-type mice, although for IL-6, the difference did not reach statistical significance (P = 0.07). Serum adiponectin, an anti-inflammatory hormone produced by adipocytes, was lower in db/db than in wild-type mice.
Genotype | Wild Type | db/db  
--- | --- | ---
IL-6, pg/ml | 23.6±2.8 | 37.3±8.7
Monocyte chemoattractant protein-1, pg/ml | 15.4±1.6 | 30.9±2.9
Leptin, ng/ml | 4.7±0.8 | 12.6±2.7
Adiponectin, μg/ml | 17.9±2.3 | 12.4±1.6
Total blood leukocytes (×10⁶/ml) | 4.2±0.5 | 3.8±0.6
Total blood neutrophils (×10⁶/ml) | 0.60±0.15 | 0.65±0.17

Results are means ± SE of data from 9–10 mice in each group. *P < 0.05 vs. wild-type mice.

cytokines (44), was lower in db/db than in wild-type mice. In contrast to the results with Cpefat mice (36), total blood leukocytes and total blood neutrophils were not different in wild-type vs. db/db mice.

Changes in ventilation during O3 exposure. The dose of O3 delivered to the lungs is the product of O3 concentration, exposure time, and VE (60), and in mice, VE declines substantially during O3 exposure (50). Consequently, we measured VE and the pattern of breathing in db/db and wild-type mice during O3 exposure (Fig. 6). We did not observe any statistically significant differences in VE between wild-type and db/db mice before O3 exposure (Fig. 6A, time 0), although there were differences in the pattern of breathing. In particular, breathing frequency was higher in db/db (319 ± 22 breaths/min) than in wild-type mice (254 ± 14 breaths/min; P < 0.05), but VT was not significantly different (0.22 ± 0.02 and 0.24 ± 0.01 ml in db/db and wild-type mice, respectively). As previously reported (50), there was a marked decrease in VE during O3 exposure in wild-type mice (Fig. 6A). Db/db showed a similar pattern of response, and although the differences were not statistically significant, VE tended to be higher in db/db than in wild-type mice at all times during O3 exposure, so that when the total volume of air inhaled during the 3-h O3 exposure was computed for each group, the difference was statistically significant (6.75 ± 0.94 and 9.63 ± 0.91 l for wild-type and db/db mice, respectively; P < 0.05). These results indicate that the total inhaled dose of O3 was greater for db/db than for wild-type mice, which could potentially account for at least part of the observed differences in response to O3 between these two genotypes (Figs. 1, 3, and 5). Changes in breathing pattern that were induced by O3 were also different in db/db mice compared with wild-type mice. In particular, repeated-measures ANOVA indicated that the striking increase in EEP that is characteristic of mice exposed to O3 (50) was significantly blunted in db/db mice (P < 0.001; Fig. 6B).

DISCUSSION

Our results indicate that compared with wild-type mice, db/db mice have small lungs, innate nonspecific AHR, greater increases in airway responsiveness and airway inflammation after O3 exposure, and reduced ventilatory responses to O3. This phenotype is qualitatively similar to that exhibited by ob/ob mice. Nevertheless, there were differences in the magnitude of O3-induced pulmonary cytokine and chemokine expression and in neutrophil recruitment to the lung between db/db and ob/ob mice. In the ensuing discussion, we describe the implications of these similarities and differences, which are summarized in Table 2.

Our results indicate that db/db mice exhibit innate AHR (Fig. 1). Innate AHR is also observed in two other types of obese mice, ob/ob mice (46, 50) and Cpefat mice (36), indicating that it is a common feature of murine obesity. Our data also extend results in other types of murine obesity by indicating that AHR is observed not only when MCh is used as the bronchoconstricting agent but also with 5-HT. Nonspecific AHR to multiple agonists is also a feature of human asthma. An association between obesity and AHR has also been reported in three large epidemiological studies (13, 15, 39), although this association has not been consistently observed (21).

Potential explanations for the innate AHR observed in obese mice have been previously discussed (36, 46, 48–50) and include differences in tidal stretching of the airway smooth muscle, reduced lung volume, effects of obesity on lung and airway development, and systemic inflammation. We have ruled out differences in tidal stretching as a potential explanation because measurements of lung mechanics were made with the mice mechanically ventilated at a fixed VT and a fixed PEEP. With respect to lung volume, it is known that airway responsiveness increases when subjects breathe at low lung volumes.
AHR, airway hyperresponsiveness; O3, ozone; BAL, bronchoalveolar lavage; EEP, end-expiratory pause; WT, wild type; MIP-2, macrophage inflammatory protein-2; sTNFR1, soluble TNF receptor 1.

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<tr>
<th>Innate AHR</th>
<th>Ob/ob Mice</th>
<th>Db/db Mice</th>
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<tr>
<td>Small lungs</td>
<td>Yes</td>
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<tr>
<td>O3-induced AHR</td>
<td>Greater than WT</td>
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<td>O3-induced BAL Cpefat</td>
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<td>O3-induced BAL IL-6</td>
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<td>O3-induced BAL KC</td>
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<td>O3-induced BAL MIP-2</td>
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<td>O3-induced BAL neutrophils</td>
<td>Greater than WT</td>
<td>Greater than WT</td>
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<tr>
<td>O3-induced BAL sTNFR1</td>
<td>No change</td>
<td>Increase</td>
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<tr>
<td>Effect of O3 on pulmonary IL-1β mRNA expression</td>
<td>No change</td>
<td>Increase</td>
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<tr>
<td>Effect of O3 on pulmonary TNF-α mRNA expression</td>
<td>Less than WT</td>
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We and others have reported that in mice, acute O3-induced AHR is mechanistically linked to several aspects of O3-induced inflammation (16, 34, 45, 51), so it is likely that the increased O3-induced AHR observed in obese vs. lean mice is the result of their greater inflammatory response. We do not know precisely which aspect of the inflammatory response is important, but it appears unlikely that influx of neutrophils contributes to the greater O3-induced AHR observed in obese mice. Compared with wild-type mice, both ob/ob (50) and db/db mice (Fig. 1) had greater O3-induced AHR, but only db/db mice (Fig. 3) and not ob/ob mice (50) had greater O3-induced neutrophils. As described above, obese mice (29, 36), like obese humans (10, 28), have low-grade chronic systemic inflammation. In humans, obesity-related systemic inflammation is likely functionally important, as it correlates with diseases of the metabolic syndrome including Type 2 diabetes and atherosclerosis (5, 58). Thus it is possible that the greater pulmonary inflammatory response to O3 observed in obese mice is the result of this systemic inflammation priming the lung to respond more vigorously to subsequent inflammatory stimuli such as O3. The reduction in adiponectin (Table 1), which has anti-inflammatory properties (44), may also contribute to the increased pulmonary inflammation observed in db/db mice. Ob/ob mice (31) also have reduced adiponectin expression compared with wild-type controls. Serum adiponectin is also reduced in obese humans (30).

Our results indicate that compared with wild-type mice, db/db have small lungs. Displacement lung volume at FRC was significantly lower in db/db than in wild-type mice. Similar results are obtained in ob/ob mice (54), and in these mice, the reduction in lung volume appears to be the result of decreased lung growth, since lung mass is also reduced (50). The reason is not yet known. It could result from the lack of leptin, which may promote lung growth (6, 57). If so, the observation that...
both db/db and ob/ob mice display this characteristic suggests that it is mediated by leptin acting via the Ob-Ra. The observation that lung size is normal in obese Cpeαα mice, which do not lack leptin, supports this hypothesis (36). Alternatively, increased abdominal fat mass may restrict lung growth during development: ob/ob and db/db mice are obese very early in development, when their lungs are still growing.

The discussion of our results up to this point has highlighted similarities between ob/ob and db/db mice. There were also notable differences. O3-induced increases in BALF neutrophils and MIP-2 were greater in db/db vs. wild-type mice but not in ob/ob vs. wild-type mice (Fig. 3). Lung IL-1β and TNF-α mRNA expression were significantly increased by O3 in db/db mice, whereas this was not the case for ob/ob mice (Fig. 5). This is the first report of differences in the pulmonary phenotype between db/db and ob/ob mice. Other differences between ob/ob and db/db mice have been reported and have been attributed to effects of leptin acting via short forms of the leptin receptor (17, 20, 33, 47). Our data confirm that Ob-Rα is expressed in the lungs of both wild-type and db/db mice. Because serum leptin concentrations are extremely high in db/db mice (Table 1), and since the augmentations in O3-induced BALF neutrophils and MIP-2 obtained in db/db mice mimic those obtained in Cpeαα mice in which leptin is also substantially elevated (36), it is possible that the observed differences between ob/ob and db/db mice are the result of leptin at high concentrations signaling through the short forms of the receptor in the db/db mouse. In contrast, ob/ob mice are devoid of any leptin signaling. In this respect, the db/db mouse may be a better model for human obesity than the ob/ob mouse, since human obesity is characterized by increases in serum leptin (18).

We do not know the precise cell or tissue that is the target for the apparent actions of leptin in the db/db mouse. Ob-Rα is expressed both in the brain and in peripheral tissues including the lung (26, 41). Indeed, Ob-Rα expression is higher in lung tissue than in any other organ including the brain (41). Macrophages express Ob-Rα receptors (22) and respond to leptin with increased production of proinflammatory cytokines (40). Because alveolar macrophages are likely an important source of the IL-1β and TNF-α that are induced following inhalation of O3 (3), the O3-induced increases in IL-1β and TNF-α mRNA expression observed in db/db but not ob/ob mice (Fig. 5) may have been the result of leptin acting via Ob-Rα receptors on lung macrophages. Data from our lab indicate that IL-1 is required for O3-induced increases in BAL MIP-2 (unpublished observations) and that chemokines, such as MIP-2 that act through the CXCR-2 receptor, are required for O3-induced increases in BAL neutrophils (34). Thus differences in O3-induced MIP-2 and O3-induced neutrophils between ob/ob and db/db mice may be secondary to differences in IL-1β expression (Fig. 5). Leptin has also been shown to induce IL-1β expression in the brain of db/db mice (27). It is not clear whether Ob-Rα-induced ERK or phosphatidylinositol 3-kinase activation plays a role in these events, but ERK has been reported to induce activation of NF-kB activation (4), an important transcription factor in the regulation of IL-1β expression. Neutrophils express Ob-Rα but not Ob-Rβ (61), and it is possible that differences in O3-induced BAL neutrophils observed between db/db and ob/ob mice are the result of direct effects of leptin acting on neutrophils via Ob-Rα. For example, leptin has been shown to inhibit neutrophil apoptosis (8).

In summary, our data indicate that db/db mice have innate, nonspecific AHR. O3 also caused more substantial increases in airway responsiveness and in BALF or lung cytokines, chemokines, protein, and neutrophils in db/db mice than in wild-type mice. Qualitatively similar results have been obtained in two other types of obese mice, ob/ob and Cpeαα mice. Because these mice differ with respect to the modality of their obesity and with respect to some other comorbidities such as hyperglycemia, the results from these three models, together, strongly support the hypothesis that obesity is responsible for this phenotype. Although db/db and ob/ob mice displayed qualitatively similar responses to O3, there were differences in the magnitude of O3-induced pulmonary cytokine expression, BAL MIP-2, and in neutrophil recruitment to the lungs. These differences suggest that leptin, acting through short forms of the leptin receptor, has the capacity to modify pulmonary responses to O3, at least when leptin is present at very high concentrations.

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