Pulmonary responses to subacute ozone exposure in obese vs. lean mice

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Shore SA, Lang JE, Kasahara DI, Lu FL, Verbout NG, Si H, Williams ES, Terry RD, Lee A, Johnston RA. Pulmonary responses to subacute ozone exposure in obese vs. lean mice. J Appl Physiol 107: 1445–1452, 2009. First published September 10, 2009; doi:10.1152/japplphysiol.00456.2009.—The purpose of this study was to determine whether obesity affects pulmonary responses following a 3-day ozone exposure. Obese db/db and lean wild-type mice were exposed to ozone (0.3 ppm) for 72 h. In wild-type mice, ozone exposure caused pulmonary injury and inflammation, and these events were associated with reduced pulmonary compliance. In db/db mice, ozone-induced neutrophil recruitment to the lung was reduced and no reduction in compliance was observed. Similar results were obtained in obese Cpefat mice, indicating that loss of leptin signaling in db/db mice does not account for these obesity-related changes. To examine the role of interleukin (IL)-6 in this obesity-related difference in ozone responsiveness, wild-type and IL-6-deficient mice were raised on 10% or 60% fat diets. Compared with 10% fat-fed mice, wild-type 60% fat-fed mice were obese and had reduced neutrophil recruitment following ozone. IL-6 deficiency reduced ozone-induced neutrophil recruitment in 10% fat-fed mice. In contrast, in obese mice, no effect of IL-6 deficiency on neutrophil recruitment was observed. Obesity-related differences in the effect of ozone on compliance were observed in both wild-type and IL-6-deficient mice. Obesity-related differences in serum IL-6 were observed and accounted for obesity-related differences in the effect of IL-6 deficiency on neutrophil recruitment. In summary, the neutrophilic inflammation induced by prolonged low level ozone exposure was attenuated in obese mice and appeared to result from an absence of IL-6-dependent neutrophil recruitment in the obese mice.

neutrophil; macrophage; sTNFR1; dynamic compliance; pulmonary resistance

Ozone (O3) is an air pollutant that increases the risk of asthma exacerbations (13, 43). Inhalation of O3 injures lung epithelial cells, causing an inflammatory response characterized by neutrophil influx and the generation of cytokines and chemokines (5, 11, 19, 24, 31, 47, 48). O3 also disrupts the epithelial barrier, leading to increased permeability and increases in bronchoalveolar lavage (BAL) protein (5, 43a).

The majority of the U.S. population is either overweight or obese. Greater decrements in lung function induced by acute O3 exposure are observed with increasing body mass index in human subjects (4). We have previously reported that the pulmonary inflammation and lung hyperpermeability induced by acute O3 exposure are also augmented in obese ob/ob (35, 38), db/db (30), and Cpefat mice (21), as well as in mice with diet-induced obesity (DIO) (20). These effects were observed following a single acute exposure to a high concentration of O3 (2 ppm) for 3 h. However, elevated atmospheric O3 tends to persist not for hours but for several days or even weeks (1), and it is not known whether similar obesity-related differences are observed with more prolonged O3 exposure. In mice, the factors that determine pulmonary responses to short-duration, high-dose O3 exposure (acute, 2 ppm for 3 h) are different from those that determine responses to longer, lower-dose O3 exposure (subacute, 0.3 ppm for 72 h) (10, 18, 19, 25, 39), suggesting that obesity might indeed have different modifying effects on pulmonary responses to subacute vs. acute O3 exposure.

Accordingly, we examined pulmonary inflammation and injury following subacute O3 exposure (0.3 ppm for 72 h) in obese db/db and lean wild-type mice. To determine the functional impact of these changes, we also examined pulmonary mechanics. In contrast to the effects of obesity on neutrophil recruitment following acute O3 exposure, we observed reduced rather than increased neutrophil recruitment following subacute O3 in db/db mice. Db/db mice are obese because of a deficiency in the receptor for leptin, a satiety hormone (8). Others have reported that the pulmonary injury and inflammation induced by chronic exposure to another oxidant, hyperoxia, are also attenuated in db/db mice (8) and that this effect appears to be related to the absence of leptin signaling (3). To determine whether the effects we observed in db/db mice were the result of obesity or the result of the inability of these mice to respond to leptin, experiments were also performed in Cpefat mice. Cpefat mice are also obese, but the leptin receptor is intact, and serum leptin is increased (21).

IL-6 is a pleiotropic cytokine that is expressed in adipose tissue of obese mice (45). Importantly, serum levels of IL-6 increase with adiposity and are associated with the development of obesity-related conditions, including cardiovascular disease and type 2 diabetes, both in humans and in animal models (2, 7, 17, 28, 33, 34, 40, 42). IL-6 is also induced in the lung following both acute and subacute O3 exposure in mice (9, 18, 19, 47, 48). We and others have shown that in lean mice, IL-6 contributes to the pulmonary injury and inflammation induced by O3 but that the role of IL-6 varies with the duration and intensity of the O3 exposure regimen (19, 48). Our data also indicate that there are differences in expression of STAT-1, a component of the IL-6 signal transduction pathway, in obese vs. lean mice (27). Hence, to determine whether IL-6 plays a role in the response to subacute O3 exposure in obese mice, we also examined wild-type or IL-6−/− mice that were rendered obese by feeding them a high-fat diet from the time of weaning.

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METHODS

Animals. The Harvard Medical Area Standing Committee on Animals approved this study. Db/db mice and Cpefat mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice are each on a C57BL/6J background, so age- and sex-matched C57BL/6J mice were used as controls. Db/db mice are obese because of a mutation in the satiety hormone, leptin (8). Cpefat mice are obese because of a mutation in the gene encoding carboxypeptidase E (Cpe), an enzyme important in processing neuropeptides involved in appetite regulation (29). Db/db mice and their wild-type controls were studied at 10–13 wk of age and were all female. Because obesity develops more slowly in Cpefat than db/db mice, male and female Cpefat mice and their wild-type controls were studied at ~24 wk of age. Db/db and Cpefat mice and their wild-type controls were fed regular mouse chow. To generate mice with DIO, litters of wild-type (C57BL/6) and IL-6 deficient (IL-6<sup>−/−</sup>) were measured using the forced oscillation technique, with a chamber. O3 was generated by passing 100% oxygen through ultra-violet (UV) light, which was subsequently mixed with room air in the chamber. O3 was generated by a UV photometric O3 calibrator (model 49PS; Thermo Electron Instruments; Hopkinton, MA), which was calibrated by a UV photometric O3 analyzer (model 49; Thermo Electron Instruments). In wild-type mice, suggesting a slightly higher metabolic rate (30). A wide incision in the chest wall was made bilaterally to expose the lungs to atmospheric pressure, and a positive end-expiratory pressure (250× magnification) for differential cell analysis. BAL supernatant was subsequently analyzed for total protein concentration using the Bradford protein assay procedure (Bio-Rad; Hercules, CA). BAL soluble TNF receptor 1 (sTNFR1), IL-6, KC, and MIP-2, and serum IL-6 were determined either by ELISA (IL-6) or with DuoSet ELISA development systems (sTNFR1, KC, MIP-2) (all R&D Systems; Minneapolis, MN).

Statistical analysis. Comparisons of pulmonary mechanics and BAL and serum parameters were assessed by factorial ANOVA, using genotypy (and/or diet) and exposure (subacute air or O3) as the main effects. Fisher’s least significant difference test was used as a follow-up test. BAL cell numbers were log transformed before analysis because the raw values were not normally distributed. STATISTICA software (StatSoft; Tulsa, OK) was used to perform all statistical analyses. The results are expressed as means ± SE, unless otherwise indicated. A P value < 0.05 was considered significant.

RESULTS

Db/db mice. Db/db mice were markedly obese (Table 1), with an average body mass ~160% more than that of their lean, wild-type controls. In air-exposed mice, baseline Rt was higher and C<sub>dyn</sub> was lower in db/db mice compared with their lean, wild-type controls (Table 1), as previously reported (30). Compared with air exposure, subacute O3 exposure caused a significant decrease in C<sub>dyn</sub> in wild-type mice. In contrast, there was no significant decrease in C<sub>dyn</sub> in db/db mice on O3 exposure. Factorial ANOVA indicated that subacute O3 exposure caused an increase in BAL macrophages and BAL neutrophils (Fig. 1). There was no significant difference between wild-type and db/db mice in the magnitude of the O3-induced change in BAL macrophages (Fig. 1A). Note that the total number of macrophages was less in db/db than wild-type mice regardless of exposure (Fig. 1A). This decrease is likely a reflection of lung size, which is substantially reduced in db/db mice (30). However, BAL neutrophils were significantly reduced in db/db vs. wild-type mice exposed to O3 (Fig. 1B). O3 was collected and stored at −80°C until further use, and the remaining cell pellet was resuspended in 1 ml of Hanks’ balanced salt solution. The total number of BAL cells was determined by manual counting with a hemacytometer. In addition, an aliquot of cells was spun onto glass microscope slides at 800 rpm for 10 min at room temperature using a Cytospin 3 Cytocentrifuge (Thermo Shandon; Pittsburgh, PA). Slides were air-dried, stained with Hema 3 (Biochemical Sciences, Swedenboro, NJ), and at least 300 cells counted under a light microscope for differential cell analysis. BAL supernatant was subsequently analyzed for total protein concentration using the Bradford protein assay procedure (Bio-Rad; Hercules, CA). BAL soluble TNF receptor 1 (sTNFR1), IL-6, KC, and MIP-2, and serum IL-6 were determined either by ELISA (IL-6) or with DuoSet ELISA development systems (sTNFR1, KC, MIP-2) (all R&D Systems; Minneapolis, MN).

Table 1. Body mass and baseline pulmonary mechanics of lean, wild-type (C57BL/6J), and obese mice exposed to air or O3 (0.3 ppm for 72 h)

<table>
<thead>
<tr>
<th>Mouse (Exposure)</th>
<th>Body Mass, g</th>
<th>Rt, cmH&lt;sub&gt;2&lt;/sub&gt;O·ml&lt;sup&gt;−1&lt;/sup&gt;·s&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>C&lt;sub&gt;dyn&lt;/sub&gt;, ml/cmH&lt;sub&gt;2&lt;/sub&gt;O</th>
<th>Cpefat&lt;sup&gt;−/−&lt;/sup&gt; (air)</th>
<th>Cpefat&lt;sup&gt;−/−&lt;/sup&gt; (O3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (air)</td>
<td>19.1±0.5</td>
<td>0.59±0.02</td>
<td>0.038±0.001</td>
<td>0.026±0.015</td>
<td></td>
</tr>
<tr>
<td>Wild type (O3)</td>
<td>19.3±0.7</td>
<td>0.58±0.01</td>
<td>0.034±0.001*</td>
<td>0.026±0.001†</td>
<td></td>
</tr>
<tr>
<td>Db/db (air)</td>
<td>50.2±1.2†</td>
<td>0.74±0.02†</td>
<td>0.037±0.001†</td>
<td>0.027±0.001†</td>
<td></td>
</tr>
<tr>
<td>Db/db (O3)&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>50.1±1.2†</td>
<td>0.72±0.03†</td>
<td>0.047±0.002*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (air)</td>
<td>30.7±2.1</td>
<td>0.54±0.015</td>
<td>0.038±0.002*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (O3)</td>
<td>35.6±2.7</td>
<td>0.63±0.038*</td>
<td>0.036±0.002†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cpefat&lt;sup&gt;−/−&lt;/sup&gt; (air)</td>
<td>59.1±1.9†</td>
<td>0.67±0.015†</td>
<td>0.036±0.002†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Cpefat<sup>−/−</sup> (O3) | 60.2±2.2† | 0.69±0.026       | 0.033±0.002     

Results are means ± SE. O3, ozone; Rt, pulmonary resistance; C<sub>dyn</sub>, dynamic compliance. *P < 0.05 compared with age- and sex-matched air-exposed mice of the same genotype. †P < 0.05 compared with age- and gender-matched wild-type mice with an identical exposure.
OBESITY AND OZONE: ROLE OF IL-6

Fig. 1. Bronchoalveolar lavage (BAL) macrophages (A), neutrophils (B), protein (C), and soluble TNF receptor (sTNFR1; D) in db/db and wild-type (WT) mice exposed to room air or to ozone (O3) (0.3 ppm) for 72 h. Results are means ± SE; n = 4–12 mice in each group. *P < 0.05 compared with genotypematched, air-exposed controls; #P < 0.05 compared with wild-type mice with the same exposure.

causes disruption of the pulmonary epithelial barrier leading to increased permeability, permitting accumulation of serum proteins into the air spaces (5). Changes in total BAL protein are often used to assess this barrier injury (5, 43a). In wild-type mice, subacute O3 exposure increased BAL protein (Fig. 1C), consistent with previous reports by ourselves and others (10, 18, 19, 24). O3-induced changes in BAL protein were significantly lower in db/db than in wild-type mice. Indeed, O3 exposure had no significant effect on BAL protein in db/db mice. We also measured BAL concentrations of sTNFR1, the extracellular domain of the p55 TNF receptor (TNFR1) that is released from cell membranes by proteolytic cleavage (Fig. 1D). Subacute O3 exposure caused a significant increase in BAL sTNFR1 in wild-type mice, consistent with previous reports (18, 19) (Fig. 1D). In contrast, no significant change in BAL sTNFR1 was observed in db/db mice.

Cpefat mice. To determine whether the effects observed in db/db mice were the result of obesity or the result of leptin receptor deficiency, experiments were repeated in Cpefat mice. Cpefat mice weighed ~80% more than their wild-type controls (Table 1). Baseline Rl was higher and Cdyn was lower in Cpefat vs. wild-type mice (Table 1), similar to db/db mice. Compared with air exposure, subacute O3 exposure caused a significant decrease in Cdyn of ~19% in wild-type mice (P < 0.05) (Table 1). In contrast, O3 exposure caused only an 8% decrease in Cdyn in Cpefat mice, which was not significantly different from 0. Similar to the results obtained in db/db mice, there was no significant difference between wild-type and Cpefat mice in the magnitude of the O3-induced increase in BAL macrophages (Fig. 2A). O3 increased BAL neutrophils in both wild-type and Cpefat mice, but the effect was significantly reduced in Cpefat mice (Fig. 2B). O3-induced increases in BAL sTNFR1 were also significantly reduced in Cpefat vs. wild-type mice exposed to O3 (Fig. 2D). O3-induced changes in BAL protein in wild-type vs. Cpefat mice were not significantly different (Fig. 2C).

To examine the time course of O3-induced responses, we repeated these experiments in another cohort of wild-type and Cpefat mice exposed to O3 (0.3 ppm) for 12, 24, or 48 h (Fig. 3). Factorial ANOVA using exposure time and genotype as main effects indicated a significant effect of genotype on BAL macrophages, neutrophils, and protein (P < 0.05 in each case). For macrophages and protein, the responses were greater in the Cpefat than in the wild-type mice. A similar trend was observed for BAL sTNFR1, but the effect did not reach statistical significance (P = 0.085). In contrast, BAL neutrophils were lower in Cpefat vs. wild-type mice throughout the exposure, and the effect became significant at 48 h of O3. We were unable to detect any O3-induced changes in BAL KC or MIP-2 in either wild-type or Cpefat mice.

DIO and the role of IL-6. To examine the role of IL-6 in subacute responses to O3 in obese mice, we repeated the 72 h experiments in wild-type and IL-6−/− mice raised on diets in which 10% or 60% of calories were derived from fat in the form of lard. There was a significant effect of diet (P < 0.001) on body weight but no effect of genotype: both wild-type and IL-6−/− mice raised on diets in which 60% of calories derived from fat weighed ~60% more than mice raised on diets in which only 10% of the calories derived from fat (Fig. 4A). There was also an interaction between diet and exposure. Mice raised on 10% fat diets had a small decline in body weight with O3 exposure, whereas this was not the case in mice with DIO (Fig. 4A).

Factorial ANOVA indicated a significant effect of exposure (P < 0.05), but not of genotype or diet on baseline Rl. The
exposure effect lay in the mice raised on 60% fat diets in which O₃ resulted in a significant decrease (P < 0.05) in baseline Rl, whereas there was no effect of exposure in the 10% fat fed mice (Fig. 4B). Factorial ANOVA indicated no significant effect of diet, genotype, or exposure on baseline Cdyn. However, there was a significant interaction between diet and exposure (P < 0.002). Compared with air exposure, subacute exposure to O₃ resulted in a significant (P < 0.05) decrease in Cdyn in mice raised on 10% fat diets (Fig. 4C), consistent with results in wild-type mice raised on standard mouse chow (Table 1). In contrast, in mice raised on 60% fat diets, no such reduction in Cdyn was observed (Fig. 4C), similar to the results with db/db and Cpefat mice (Table 1).

Factorial ANOVA indicated a significant effect of exposure (P < 0.01) and of diet (P < 0.05) on BAL macrophages, but no overall effect of genotype. However, there was a significant interaction between genotype and diet (P < 0.01). In wild-type mice raised on 10% fat diets, subacute O₃ exposure caused an increase in macrophages (Fig. 5A). In contrast, no significant increase in BAL macrophages was observed in wild-type mice raised on 60% fat diets. IL-6 deficiency ablated the effect of O₃ on BAL macrophages in lean mice, but IL-6 deficiency did not affect BAL macrophages in O₃-exposed obese mice (Fig. 5A). There was also a significant effect of exposure on BAL neutrophils (P < 0.001), as well as a significant interaction between exposure, diet, and genotype (P < 0.002). Compared with air, O₃ exposure increased BAL neutrophils regardless of whether the mice were raised on 10% or 60% fat diets, although the impact of O₃ on BAL neutrophils was significantly less (P < 0.01) in the 60% vs. the 10% fat-fed mice (Fig. 5B), similar to the results described above in db/db and Cpefat mice. IL-6 deficiency resulted in a significant (P < 0.01) reduction in BAL neutrophils in the lean mice, but not the obese mice (Fig. 5B), such that in IL-6 deficient mice, there was no difference in BAL neutrophils between obese and lean O₃ exposed mice.

There was a significant effect of O₃ exposure on BAL protein (P < 0.001); compared with air, O₃ exposure increased BAL protein in all types of mice examined, whether they were lean or obese and whether they were wild type or IL-6 deficient (Fig. 5C). However, there was no significant effect of diet on BAL protein. Factorial ANOVA also indicated a significant effect of both diet (P < 0.05) and exposure (P < 0.001) on sTNFR1 (Fig. 5D). Ozone caused an increase in BAL sTNFR1 in both 10% fat-fed (P < 0.01) and 60% fat-fed (P < 0.01) mice and in both wild-type (P < 0.01) and IL-6-deficient (P < 0.05) mice. However, the increase in sTNFR1 induced by O₃ was reduced (P < 0.05) in IL-6 vs. wild-type mice. When considered without regard to exposure we also observed a significant increase in BAL sTNFR1 in obese vs. lean wild-type mice (P < 0.05), whereas this obesity-related difference was abolished in IL-6-deficient mice.

Effects of O₃ exposure and obesity on IL-6. To examine O₃- and obesity-induced changes in IL-6, we measured BAL and serum IL-6 by ELISA in mice with DIO and their lean controls, and in Cpefat mice and their wild-type controls exposed to O₃ for 12, 24, or 72 h (Fig. 6). IL-6 values of IL-6−/− mice (10% fat, air exposed) are included as negative controls. In the DIO mice and their lean controls, factorial ANOVA indicated a significant effect of O₃ exposure, but not diet (10% vs. 60% fat) on BAL IL-6 (Fig. 6A). To determine whether there might be differences in the kinetics of O₃-induced increases in BAL IL-6 in lean vs. obese mice, we also examined BAL IL-6 in the Cpefat and wild-type mice that we had exposed for 12, 24, or 48 h (Fig. 6B). Factorial ANOVA indicated that O₃ caused a time-dependent increase in BAL IL-6 (P < 0.01), but there was no effect of obesity on BAL IL-6. In air-exposed mice, serum IL-6 was greater in mice with DIO than in 10% fat-fed controls (Fig. 6C), although this difference was no longer apparent by 72 h of exposure. Factorial ANOVA indicated a significant effect of obesity (P < 0.05) but no exposure time on serum IL-6 in Cpefat vs. wild-type mice exposed to O₃ for 12, 24, or 48 h. Serum IL-6 was greater in obese Cpefat mice than their lean wild-type controls (Fig. 6D).

**DISCUSSION**

Our results indicate that in wild-type mice, subacute O₃ exposure (0.3 ppm for 72 h) causes pulmonary injury and inflammation, and these events are associated with reduced dynamic compliance of the lung. In obese mice, O₃-induced neutrophil emigration into the lungs was markedly attenuated, and in these mice, no reduction in lung compliance was observed. These reduced responses to subacute O₃ exposure were observed regardless of the cause of the obesity. The obesity-related difference in O₃-induced neutrophil influx was dependent on IL-6: in lean but not obese mice, IL-6 deficiency caused a marked decrease in neutrophil influx. Consequently, the greater O₃-induced neutrophil influx observed in lean vs. obese wild-type mice was no longer observed when the mice were IL-6 deficient.
In wild-type mice, subacute O₃ exposure caused the typical pattern of pulmonary inflammation and injury. Both BAL macrophages and neutrophils increased (Figs. 1, 2, 3, 5), consistent with previous reports by ourselves and others in mice exposed in the same manner (10, 18, 24). Subacute O₃ exposure also caused an increase in BAL protein (Figs. 1, 2, 5). Others have shown that O₃ causes disruption of the pulmonary epithelial barrier leading to increased permeability, resulting in the accumulation of serum proteins in the air spaces (5, 43a), including after subacute O₃ exposure (10, 18, 19, 24). O₃ exposure (0.3 ppm for 72 h) also caused a significant decrease in Cdyn in wild-type mice (Table 1), consistent with inhomogeneous small airway narrowing. sTNFR1 increased following subacute O₃ exposure in wild-type mice (Table 1), consistent with inhomogeneous small airway narrowing. sTNFR1 increased following subacute O₃ exposure in wild-type mice (Figs. 1, 2, 5), consistent with previous reports (18, 19). sTNFR1 is the extracellular domain of the p55 TNF receptor and is released from the cell surface by proteolytic cleavage by TNF-α converting enzyme (TACE). Other stimuli that induce reactive oxygen species also cause shedding of TNF receptors from pulmonary epithelial cells in culture (15).

O₃-induced increases in BAL neutrophils were markedly attenuated in obese db/db mice (Fig. 1). This reduction in neutrophils was not the result of reduced blood leukocytes: we have previously reported similar blood leukocytes in db/db vs. wild-type mice (30). Similarly, O₃-induced increases in total BAL protein and in BAL sTNFR1 were attenuated in db/db mice (Fig. 1), and O₃-induced reductions in Cdyn were abolished. Pulmonary injury and inflammation induced by chronic exposure to another oxidant, hyperoxia, are also attenuated in db/db mice (3). Since intratracheal instillation of leptin can also cause lung injury, the authors of that study concluded that the relative protection from the effects of hyperoxia in db/db mice may arise from their deficiency in leptin signaling (3). We have previously reported that leptin also alters responses to acute O₃ under some conditions (22, 38). Hence, to determine whether alterations in leptin might explain the reduced responses of db/db mice to subacute O₃, we repeated the experiments in Cpefat mice. These mice have intact leptin receptors and increased serum leptin (21). However, the responses of Cpefat mice to subacute O₃ were essentially similar to those obtained in db/db mice: O₃-induced neutrophil influx and O₃-induced reductions in Cdyn were attenuated in Cpefat vs. wild-type mice (Fig. 2, Table 1). This was not the result of differences in the kinetics of neutrophil recruitment between the obese and lean mice, since essentially similar results were obtained throughout the exposure (Fig. 3B). Similar results were also observed in mice with DIO vs. lean controls (Fig. 5B). These mice also have intact leptin receptors and increased serum leptin (20). The results suggest that obesity, rather than leptin receptor deficiency, accounts for these differences in O₃-induced neutrophil migration into the lungs observed in db/db mice.

In contrast to the changes in neutrophil migration, the reduction in O₃-induced lung hyperpermeability (as indicated by changes in BAL protein) that we observed in db/db mice (Fig. 1C) may indeed be related to leptin receptor deficiency,
since it was observed in db/db mice, but not in Cpefat mice (Fig. 2C) or mice with DIO (Fig. 5C). In fact, when we examined the time course of O₃-induced changes in lung hyperpermeability (Fig. 3C), we observed that the effects of O₃ were more rapid in the Cpefat mice vs. wild-type mice: maximal effects of O₃ on BAL protein occurred after only 12 h in the Cpefat mice, whereas BAL protein continued to increase with increasing duration of exposure in the wild-type mice (Fig. 3C), so that after 72 h the BAL protein responses in wild-type and Cpefat mice were the same (Fig. 2C). Similarly, BAL macrophages were actually greater in Cpefat mice than wild-type mice during the first 48 h of O₃ exposure (Fig. 3A). Taken together with the data from 72 h of exposure (Fig. 2, A and C), the results suggest that for these aspects of the response to 0.3 ppm O₃, BAL neutrophils and protein (BAL macrophages and protein), the Cpefat mice may actually be initially responding to a greater extent than wild-type mice, similar to what occurs with acute O₃ exposure (21), but then adapting over time. In contrast, neutrophil migration induced by 0.3 ppm O₃ was reduced in Cpefat mice after all durations of exposure examined (Figs. 2B and 3B).

We were quite surprised by the apparent protective effect of obesity on the neutrophil response to subacute O₃ exposure, since we have previously reported increased BAL neutrophils in Cpefat and db/db mice following an acute exposure to 2 ppm O₃ (21, 30). While the discordance between the effect of obesity on responses to acute vs. subacute exposures was unexpected, differences in the factors determining cellular inflammatory responses to acute vs. subacute O₃ exposure are not without precedent. Using recombinant inbred lines crossed from C57BL/6J and C3H/HeJ mice, Kleeberger et al. (25) demonstrated that the genetic factors that control neutrophil influx induced by subacute vs. acute O₃ are not the same. In addition, genetic deficiency in TNF receptors or anti-TNF antibodies attenuates the neutrophil influx induced by subacute but not acute O₃ exposure (10, 26, 39), and IL-1RI deficiency attenuates the neutrophil influx induced by subacute but not acute O₃ exposure (18). However, it is important to reiterate that the exposure duration experiments (Fig. 3) suggest that these differences in the impact of obesity on responses to high-dose (2 ppm) vs. low-dose (0.3 ppm) O₃ were not observed for other outcome indicators (e.g., BAL protein). Cpefat mice initially responded to 0.3 ppm O₃ with greater increases in BAL protein than wild-type mice (Fig. 3C), similar to their greater increases in BAL protein after a 3-h 2 ppm exposure (21).

It is unlikely that differences in the delivery of O₃ could account for the reduced neutrophil recruitment observed in the obese mice. The inhaled dose of O₃ is the product of O₃ concentration, exposure duration, and minute ventilation (46). However, we have reported that there are no differences in minute ventilation between db/db, Cpefat, and DIO mice and their respective lean controls (37).

Since we have previously reported that IL-6 contributes to the neutrophil influx induced by subacute O₃ exposure in the lung (19), we used IL-6-deficient mice to examine the importance of IL-6 in the differences in the cellular inflammatory response to O₃ observed between obese and lean mice. Con-

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Fig. 5. BAL macrophages (A), neutrophils (B), protein (C), and sTNFR1 (D) in mice reared on either 10% or 60% fat diets. Both wild-type (WT) and IL-6 deficient (IL-6−/−) mice on each diet were exposed to room air or to ozone (O₃) (0.3 ppm) for 72 h. Results are means ± SE; n = 5–12 mice in each group. *P < 0.05 compared with diet- and genotype-matched, air-exposed controls; #P < 0.05 compared with wild-type mice with the same diet and exposure; %P < 0.05 compared with 10% fat-fed mice with the same genotype and exposure.

Fig. 6. BAL (A), serum (C, D) concentrations of IL-6 in wild-type mice reared on diets containing either 10% or 60% fat and exposed to room air or to ozone (O₃) (0.3 ppm) for 72 h (A, C) and in Cpefat mice and their wild-type controls exposed to O₃ (0.3 ppm) for 12, 24, or 48 h. In A and C, data from IL-6−/− air-exposed mice raised on 10% fat diets are included as negative controls. Results are means ± SE; n = 7–8 mice in each group. See text for statistical analysis.
sistent with our previous observations, we observed a marked reduction in the neutrophil influx induced by subacute O₃ exposure in lean IL-6 deficient vs. lean wild-type mice (Fig. 4B). O₃ exposure did cause neutrophil recruitment in obese mice (Fig. 5B), but in contrast to the lean mice, we observed no reduction in this neutrophil response to O₃ in obese IL-6 deficient vs. obese wild-type mice (Fig. 5B). Importantly, the greater neutrophil influx that was observed in the lean vs. obese wild-type mice was abolished when the mice were IL-6 deficient. Thus the reduced neutrophil influx induced by O₃ in obese mice appears to be the result of obesity-related differences in either the amount or locus of IL-6 expression or in signaling pathways downstream of IL-6.

To address possible obesity-related differences in IL-6 expression, we measured BAL and serum IL-6 in the mice with DIO and in Cpefat mice (Fig. 6). Using IL-6−/− mice for comparison, we detected no IL-6 in BAL of air-exposed mice (Fig. 6A). Compared with air-exposed mice, BAL IL-6 increased in mice exposed to O₃ (Fig. 6, A and B). These results are consistent with reports by ourselves and others of a small increase in IL-6 mRNA in normal wild-type mice exposed in the same manner (9, 18, 19). However, there was no difference in BAL IL-6 in O₃-exposed obese vs. lean mice (Fig. 6, A and B).

While there were no obesity-related differences in BAL IL-6, there were obesity-related differences in serum IL-6 (Fig. 6, C and D). Notably, serum IL-6 was elevated in air-exposed mice with DIO (Fig. 6B). Others have reported increased adipose tissue IL-6 expression (2, 36), as well as increased circulating levels of IL-6 in obese mice and obese humans (32, 40, 44). Serum IL-6 was also higher in obese Cpefat mice vs. their lean controls at 12, 24, and 48 h of O₃ exposure. It is conceivable that obesity-related elevations in serum IL-6 contribute to obesity-related differences in the impact of IL-6 deficiency on neutrophil responses to subacute O₃ in the absence of differences in BAL IL-6 (Fig. 6, A and B) elevations in circulating IL-6 would reduce the overall lung to blood gradient for IL-6 and could thus reduce the impact of IL-6 on neutrophil migration. Elevations in serum IL-6 could also act to prevent neutrophil migration into the lungs in obese mice by increasing neutrophil adherence to systemic vascular beds.

We cannot rule out the possibility that disrupted IL-6 signal transduction was the cause of the reduced effect of IL-6 deficiency in the obese vs. lean mice (Fig. 5B). STAT1 and STAT3 are two of the major transcription factors that mediate the effects of IL-6 (14). We have previously reported reduced STAT1 expression in the lungs of obese mice (27). Furthermore, both IL-6 and leptin can induce expression of SOCS3 (23), an inhibitor of STAT3 signaling (12), and both IL-6 and leptin are elevated in the serum of mice with DIO (Fig. 6 and Refs. 20, 32).

Our data suggest that changes in soluble TNFR receptors may also play a role in the differential effects of O₃ on neutrophil influx in lean vs. obese mice. The soluble TNFR receptor, sTNFR1, is the extracellular domain of the p55 TNF receptor and is released from the cell surface by proteolytic cleavage by TACE (36). Regardless of exposure, we observed an increase in BAL sTNFR1 in mice raised on 60% vs. 10% fat diets (Fig. 5D). We observed a similar increase in BAL sTNFR1 in air-exposed Cpefat mice (Fig. 2D) and db/db mice (Fig. 1D) consistent with previous reports (21, 30). TNF-α is required for the neutrophil influx that occurs following subacute O₃ exposure in mice (10, 26). Hence, it is conceivable that elevated levels of soluble TNF receptors in BAL fluid of obese mice early in the exposure neutralizes more TNF-α, leading to reduced neutrophil influx at subsequent time points. In this respect, it is important to note that the neutrophil migration that occurs following acute rather than subacute O₃ exposure is not dependent on TNF-α (10, 39), which may explain part of the differential effects of obesity on neutrophil responses to acute vs. subacute O₃.

As discussed above, the effects of subacute O₃ exposure in mice with dietary obesity (Figs. 4 and 5) resembled in many respects the results obtained in obese db/db and Cpefat mice (Table 1, Figs. 1 and 2). However, there were some differences. Notably, the increase in BAL macrophages that occurred with subacute O₃ exposure was not different in wild-type vs. db/db or Cpefat mice, whereas O₃ exposure failed to increase BAL macrophages in mice with DIO, but did increase BAL macrophages in 10% fat-fed controls (Fig. 5A). Similar to the results with neutrophils (Fig. 5B), this obesity-related difference was ablated in IL-6−/− mice.

The mice with DIO were more than 30 wk old. The use of these older mice was necessary because the pulmonary phenotype of DIO mice requires extended periods of high-fat diet feeding to develop (20). The advanced age of the DIO mice and their 10% fat-fed controls may explain their greater baseline pulmonary compliance (compare Table 1 and Fig. 4C, air-exposed mice), since their lungs were likely bigger. Paradoxically, we also observed a reduction in Rₜ and an increase in Cdyn after O₃ exposure in the wild-type mice with DIO (Fig. 4). We do not know why these changes occurred, but these data are consistent with the hypothesis that O₃-induced activation of the sympathetic nervous system occurs in these older obese mice, thereby promoting bronchodilation.

In summary, the neutrophilic inflammation induced by prolonged low-level O₃ exposure, such as might occur in individuals living in highly polluted cities, was markedly attenuated in obese mice. This reduction in O₃-induced neutrophil influx was the result of a reduction in IL-6 driven neutrophil recruitment in the obese mice.

REFERENCES


