Pulmonary responses to acute ozone exposure in fasted mice: effect of leptin administration

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OZONE (O₃) is a common air pollutant and an asthma trigger. Emergency room visits and hospital admissions for asthma increase on days of high ambient O₃ concentrations (10, 48). Even O₃ concentrations below the current US Environmental Protection Agency standard are sufficient to initiate symptoms in children with asthma (13). Exposure to O₃ causes lung injury and inflammation, including the generation of numerous cytokines, chemokines, and eicosanoids (14, 16–19, 30, 43). Cellular changes include sloughing of epithelial cells and cytokines, chemokines, and eicosanoids (14, 16–19, 30, 43). O₃ exposure can also cause airway hyperresponsiveness (AHR), a defining feature of asthma (7, 43, 52). O₃-induced AHR and/or inflammation are likely to contribute to the ability of O₃ to initiate asthmatic episodes.

Leptin is an adipocyte-derived hormone that promotes satiety and increases metabolism. Leptin is also a member of the IL-6 family of cytokines with JAK/STAT signaling capabilities and has proinflammatory effects (47), including augmentation of cytokine release from LPS-stimulated macrophages (12, 24), increased proliferative responses to mitogenic stimuli, and increased production of Th1 cytokines in T cells (25, 28) and activation of NF-κB in endothelial cells (5).

Leptin also enhances O₃-induced pulmonary inflammation. For example, administration of exogenous leptin before and after cessation of O₃ exposure augments O₃-induced increases in bronchoalveolar lavage (BAL) protein, KC, and IL-6, in wild-type mice (43). In obese mice, increases in endogenous leptin, acting through short forms of the leptin receptor, also appear to contribute to increased O₃-induced pulmonary IL-1β mRNA expression and increased neutrophil influx into the lung (26). Leptin is increased in the obese state, and such changes may be relevant to the increased incidence and severity of asthma observed in obesity (41).

Although increases in leptin appear to increase inflammatory responses to O₃ (43), it is not known whether reductions in endogenous levels can attenuate pulmonary effects of O₃. Administration of exogenous leptin also enhances allergen-induced AHR in mice (45), but there are no data describing the effect of reductions in endogenous leptin on airway responsiveness. To examine the effects of reductions in serum leptin on O₃-induced airway inflammation and O₃-induced changes in airway responsiveness, we fasted mice before and after O₃ exposure. When mice are fed ad libitum, serum leptin levels are proportional to body mass index; however, during fasting, serum leptin levels undergo a rapid and marked decline, disproportionate to declines in body mass (1, 2, 27, 31). To determine whether the effects of fasting were the result of declines in serum leptin, we reconstituted leptin in fasted animals.

METHODS

Animals. All of the experimental procedures used in this study were approved by The Harvard Medical Area Standing Committee on Animals. Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were studied at ~8 wk of age.

Protocol. In the first series of experiments, mice were implanted with Alzet micro-osmotic pumps (model 1007D; DURECT, Cupertino, CA), which delivered sterile recombinant murine leptin (R&D Systems, Minneapolis, MN) on day 1, as described below. Leptin was dissolved with 15 mM HCl and 7.5 mM NaOH in PBS, and this was used as the vehicle control (referred to subsequently as PBS). At 3:00 PM on day 5, food was removed from the cages of one-half of the mouse population in each group. The other mice continued to have free access to food, except during O₃ exposures. All mice had free access to water except during O₃ exposures. At ~9:00 AM on the...
morning of day 6, mice in each group were exposed to either O₃ (2 ppm) or room air for 3 h. Twenty-four hours after exposure, mice were anesthetized and instrumented for the measurement of pulmonary mechanics by the forced oscillation technique: airway responsiveness to intravenous acetyl-β-methylcholine chloride (methacholine; MCh) (Sigma-Aldrich, St. Louis, MO) was then measured. Food and water were withheld after the last exposure to room air or to ozone (O₃; 2 ppm for 3 h). Results are means ± SE of data from 5–12 mice in each group. *P < 0.05 compared with fed, PBS-treated, air-exposed mice.

**RESULTS**

**Effect of fasting, O₃ exposure, and leptin administration on body mass and serum leptin.** In air-exposed mice, fasting caused a marked reduction in serum leptin (Fig. 1), as described by others (1, 2, 22, 31). Continuous infusion of leptin via Alzet micro-osmotic pumps prevented this decline in serum leptin. Indeed, serum leptin was not different in leptin-treated fasted mice vs. PBS-treated fed mice. Even in fed mice, exposure to O₃ caused a marked reduction in serum leptin, likely because of O₃-induced weight loss (Figs. 1 and 2). Imposed fasting tended to further reduce serum leptin levels in O₃-exposed mice, although the effect was not statistically significant. Continuous infusion of leptin prevented O₃-induced reductions in serum leptin in both fed and fasted mice.

Implantation of Alzet micro-osmotic pumps. Alzet micro-osmotic pumps were implanted subcutaneously in the intrascapular region of each mouse. The pumps infuse solutions at a rate of 0.5 μl/h for 7 days. The reservoir of each pump was preloaded with 96 μl of either sterile PBS or recombinant mouse leptin (0.9 μg/μl), resulting in a leptin infusion rate of ~0.44 μg·g⁻¹·day⁻¹. The dose of leptin was chosen to restore serum leptin of fasted animals to levels similar to, or fasted as described above. In this second cohort, BAL was performed, but we did not measure airway responsiveness. Because we observed virtually no effect of fasting in these latter experiments, we did not examine the effects of leptin reconstitution. In all cases, mice were weighed in the early morning.

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Effect of fasting, O3 exposure, and leptin infusion on baseline pulmonary mechanics and airway responsiveness to MCh. Factorial ANOVA indicated no effect of fasting or leptin administration on resistance or dynamic compliance (Cdyn) in air-exposed mice (Table 1). O3 exposure caused a significant, ~40% increase in baseline resistance (P < 0.01) and a decrease in baseline Cdyn (P < 0.01) in fasting but not in fed mice. Significant O3-induced changes in pulmonary mechanics were not observed in fasted mice that had been reconstituted with leptin.

In air-exposed mice, fasting resulted in a significant decrease in airway responsiveness, regardless of whether the mice were treated with PBS or with leptin (Fig. 3). O3 exposure increased airway responsiveness in fasted (Fig. 4B) but not in fed mice (Fig. 4A). This fed/fasted difference was observed only in PBS-treated mice. With leptin treatment, O3 exposure did not significantly increase airway responsiveness in either fed (Fig. 4C) or fasted (Fig. 4D) mice.

Effect of fasting and leptin infusion on O3-induced pulmonary inflammation and injury. O3 exposure caused a significant increase in the total number of cells recovered in the BAL fluid 24 h postexposure (P < 0.001) (Fig. 5). Increases in macrophages (P < 0.001), epithelial cells (P < 0.001), and neutrophils (P < 0.001) accounted for the increase in BAL cells, whereas there were few if any eosinophils or lymphocytes in the BAL in either air- or O3-exposed mice. There was no effect of fasting status on BAL cells in either air- or O3-exposed mice. Leptin treatment had no effect on BAL cell profile in air-exposed mice. Leptin treatment increased BAL epithelial cells in O3-exposed mice, regardless of fasting status, but had no effect on other BAL cell types.

Table 1. Baseline pulmonary mechanics

<table>
<thead>
<tr>
<th></th>
<th>RL, cmH2O ml⁻¹ s⁻¹</th>
<th>Cdyn, ml/cmH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>O3</td>
</tr>
<tr>
<td>Fed PBS</td>
<td>0.71±0.03</td>
<td>0.76±0.02</td>
</tr>
<tr>
<td>Fasted PBS</td>
<td>0.66±0.02</td>
<td>0.89±0.07*</td>
</tr>
<tr>
<td>Fed leptin</td>
<td>0.68±0.02</td>
<td>0.70±0.04</td>
</tr>
<tr>
<td>Fasted leptin</td>
<td>0.63±0.02</td>
<td>0.72±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE of data from 6 – 8 mice in each group. RL, pulmonary resistance; Cdyn, dynamic compliance; O3, ozone. *P < 0.01 compared with air-exposed animals in the same treatment group.

There were also significant increases in BAL sTNFR1 (P < 0.001), BAL sTNFR2 (P < 0.001), and total BAL protein (P < 0.001), 24 h after O3 exposure (Fig. 6), as previously described (19). There was no effect of either fasting or leptin treatment on sTNFR1 or sTNFR2 in either air- or O3-exposed mice. BAL protein was significantly increased in fasted vs. fed mice (P < 0.02) exposed to air regardless of whether they were leptin or PBS treated. In contrast, fasting had no effect on BAL protein in O3-exposed mice.

Whereas BAL neutrophils, sTNFRs, and protein continue to increase up to 24 h postexposure (20, 26), BAL cytokines and chemokines peak earlier and then decline to levels not different from air-exposed mice by 24 h. Therefore, to determine whether there was an effect of fasting on these measures of inflammation, we studied an additional cohort of fed and fasted mice euthanized 4 h after O3 exposure. Compared with air exposure, O3 exposure caused a significant increase in BAL protein, IL-6, eotaxin, MIP-2, sTNFR1, sTNFR2, neutrophils, and epithelial cells (Fig. 7). BAL MIP-2 was significantly decreased in fasted vs. fed mice; however, for all seven other inflammatory outcomes, there was no significant difference between fed and fasted mice.

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Effect of fasting, \(O_3\) exposure, and leptin infusion on serum corticosterone levels. Because imposed fasting could be expected to induce significant stress in these mice, we also analyzed serum corticosterone (Fig. 8). Factorial ANOVA indicated a significant effect of fasting status on serum corticosterone \((P < 0.005)\), but no significant effect of leptin treatment or \(O_3\) exposure was shown. On average, serum corticosterone was \(\sim 60\%\) higher in fasted than in fed mice. The lack of effect of leptin on fasting-induced changes in corticosterone (Fig. 7) is consistent with previous reports (27).

DISCUSSION

Our results indicate that fasting augmented changes in RL, \(C_{dyn}\), and airway responsiveness to intravenous MCh induced by \(O_3\) exposure in mice (Table 1, Fig. 4). Importantly, fasting resulted in a marked decrease in serum leptin, and restoration of leptin to fed levels by exogenous administration prevented these fasting-induced changes in the response to \(O_3\). In contrast, there was virtually no effect of fasting on the inflammatory response to \(O_3\) (Figs. 5–7).

To effect a reduction in leptin, we fasted mice before and during recovery from \(O_3\). In air-exposed mice, fasting caused a marked decrease in serum leptin to levels less than one-sixth of those observed in fed mice, consistent with previous reports (1, 2, 27, 31). This decline is consistent with the relatively short half-life of recombinant leptin in the mouse (37 min) (15). Implantation of Alzet micro-osmotic pumps delivering leptin at a rate of \(\sim 0.44 \, \mu g \cdot g^{-1} \cdot day^{-1}\) restored leptin levels in fasted mice to, but not above, those of fed controls. Although leptin is a satiety hormone and can induce a decrease in body mass even in lean mice (35, 36), we did not observe any effect of leptin on body mass, likely because the concentration of exogenous leptin administered was fairly low, low enough that it did not significantly increase serum leptin levels in the fed state (Fig. 1). When higher concentrations of leptin were administered, there was a decrease in body mass (data not shown). Surprisingly, we also observed a marked decrease in leptin in fed mice that we had exposed to \(O_3\) (Fig. 1). This \(O_3\)-induced reduction in leptin is likely the result of \(O_3\)-induced reductions in eating behavior, as evident by the decline in body weight observed in these mice (Fig. 2). \(O_3\)-induced declines in body weight have been reported by others (23, 51), who indicated that these declines are the result of both anorexia and cachexia. In fact, even when we did not impose fasting, mice substantially reduced their activity level after \(O_3\) exposure and did not actively seek food.

In fed mice, we observed no effect of exogenous leptin administration on airway responsiveness in either air-exposed or \(O_3\)-exposed mice (Figs. 3 and 4), and no effect of leptin on \(O_3\)-induced airway inflammation was shown (Figs. 5 and 6). We were initially surprised that leptin treatment did not increase \(O_3\)-induced pulmonary inflammation in the fed mice; our group (43) has previously reported an increase in BAL protein, IL-6, and KC in mice treated with exogenous leptin.
However, it should be noted that the leptin treatment administered to the mice in the present study did not increase serum leptin much above normal fed levels (Fig. 1), whereas in our previous study (43), leptin was administered in such a manner that serum leptin was substantially elevated. Accordingly, we cannot determine from these data whether more marked increases in leptin, such as those that are observed in obese individuals, could be expected to alter airway responsiveness.

Regardless of nutritional status, leptin did increase the number of epithelial cells shed into the airway lumen following O₃ exposure, suggesting that leptin enhances epithelial injury (Fig. 5). We do not know the mechanistic basis for this effect of leptin. It is possible that leptin acts directly on epithelial cells to augment O₃-induced cell shedding. Leptin receptors are expressed on airway epithelial cells (6, 50), but leptin appears to promote airway epithelial growth (50). It is also possible that administration of exogenous leptin for several days before O₃ exposure altered the epithelial phenotype in such a way as to make it more susceptible to O₃-induced injury.

Fasting had multiple effects on airway function. For example, fasting reduced airway responsiveness in air-exposed mice (Fig. 3). The reduction in airway responsiveness induced by fasting was not related to changes in leptin because it was observed in both leptin-treated and PBS-treated mice. One explanation for this fasting-related decrease in responsiveness is increased activation of the sympathetic nervous system: β-agonists reduce airway responsiveness not only in humans but also in mice (29). Consistent with this hypothesis, our data indicate that fasting induced an important stress response in these mice (Fig. 7). Others have described a marked reduction in serum glucose in mice fasted for a similar amount of time (27), and reductions in glucose are known to induce activation of the sympathetic nervous system (11).

Fasting also altered changes in pulmonary mechanics and airway responsiveness induced by O₃ (Table 1, Fig. 4). O₃ exposure altered the epithelial phenotype in such a way as to make it more susceptible to O₃-induced injury.

Fig. 5. Effect of fasting and leptin treatment on bronchoalveolar lavage (BAL) cells. BAL was performed 24 h after air or O₃ exposure. Results are means ± SE of data from 6–8 mice in each group. *P < 0.05 compared with PBS-treated mice in the same exposure group.

Fig. 6. Effect of fasting and leptin treatment on BAL sTNFR1, sTNFR2, and protein levels. Lavage was performed 24 h after air or O₃ exposure. Results are means ± SE of data from 6–8 mice in each group. *P < 0.05 compared with fed mice in the same treatment and exposure group.
decreased $C_{dyn}$, increased $R_t$, and increased airway responsiveness to MCh in fasted mice, whereas no such changes were observed in fed mice exposed to O$_3$ at the same time and in the same chamber. Because we measured mechanical responses to O$_3$ at only one time point, it is unclear whether these changes are the result of increased effects of O$_3$ in the fasted state or a delayed recovery from the exposure. However, it is clear that these fasting-induced changes in responses to O$_3$ were reversed when leptin was restored (Fig. 4D); i.e., leptin was protective against O$_3$-induced changes in lung function in the fasted mice.

It is unlikely that the ability of fasting to augment O$_3$-induced changes in $R_t$ and O$_3$-induced changes in airway responsiveness is secondary to a greater O$_3$-induced inflammatory response in the fasted mice. The usual pulmonary inflammatory responses to O$_3$ (increased BAL protein, cytokines, chemokines, and neutrophils) were evident in both fed and fasted mice, and there was no difference in the magnitude of these responses between these two conditions either 4 h (Fig. 7) or 24 h (Figs. 5 and 6) after O$_3$ exposure, except for a reduction in MIP-2 in fasted mice vs. fed mice 4 h after O$_3$ exposure (Fig. 7). These data also indicate that, although marked increases in serum leptin can augment O$_3$-induced pulmonary inflammation (43), reductions in serum leptin below normal endogenous levels (as occurs with fasting) do not reduce O$_3$-induced airway inflammation.

We do not know why leptin is able to reverse the effects of fasting on pulmonary mechanics and airway responsiveness, but it is possible that it is related to effects on lung surfactant. O$_3$ exposure causes surfactant dysfunction (32, 33, 38), an event that could be expected to promote closure of small airways during MCh challenge and to increase the resistance of the lung tissue. Leptin has been shown to stimulate surfactant synthesis in fetal lung cells (3, 49). Because fasting caused a marked reduction in serum leptin (Fig. 2) and because restoration of leptin with exogenous administration of leptin prevented the effects of fasting on O$_3$-induced changes in pulmonary mechanics (Fig. 4, Table 1), it is possible that O$_3$ exposure led to greater surfactant dysfunction in the fasted mice because of loss of the salutary effects of leptin on surfactant synthesis.

There are no other studies in the literature describing the effects of fasting on pulmonary responses to O$_3$. Kari et al. (21) and Elsayed (9) reported that caloric restriction for several weeks or months attenuates O$_3$-induced pulmonary inflammation in rats, likely as a result of increased synthesis of antioxidants, but neither group investigated pulmonary mechanics. Our study was different in that it involved complete caloric
restriction for a much shorter period of time and shows that, whereas caloric restriction protects against O₃-induced inflammation (9, 21), acute starvation worsens the effects of O₃ on lung function.

In summary, our results indicate that fasting augments O₃-induced changes in Rₛ, Cdyn, and airway responsiveness to intravenous MCh in mice. Our results also indicate that preventing declines in circulating leptin during fasting prevents these deleterious effects of fasting. The mechanistic basis for this protective effect of leptin in fasted mice remains to be determined but is not the result of effects on pulmonary inflammation.

GRANTS

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