Obese mice exhibit an altered behavioural and inflammatory response to lipopolysaccharide

Catherine B. Lawrence¹,*, David Brough¹ and Elysse M. Knight¹

SUMMARY

Obesity is associated with an increase in the prevalence and severity of infections. Genetic animal models of obesity (ob/ob and db/db mice) display altered centrally-mediated sickness behaviour in response to acute inflammatory stimuli such as lipopolysaccharide (LPS). However, the effect of diet-induced obesity (DIO) on the anorectic and febrile response to LPS in mice is unknown. This study therefore determined how DIO and ob/ob mice respond to a systemic inflammatory challenge. C57BL/6 DIO and ob/ob mice, and their respective controls, were given an intraperitoneal (i.p.) injection of LPS. Compared with controls, DIO and ob/ob mice exhibited an altered febrile response to LPS (100 μg/kg) over 8 hours. LPS caused a greater and more prolonged anorectic effect in DIO compared with control mice and, in ob/ob mice, LPS induced a reduction in food intake and body weight earlier than it did in controls. These effects of LPS in obese mice were also seen after a fixed dose of LPS (5 μg). LPS (100 μg/kg) induced Fos protein expression in several brain nuclei of control mice, with fewer Fos-positive cells observed in the brains of obese mice. An altered inflammatory response to LPS was also observed in obese mice compared with controls: changes in cytokine expression and release were detected in the plasma, spleen, liver and peritoneal macrophages in obese mice. In summary, DIO and ob/ob mice displayed an altered behavioural response and cytokine release to systemic inflammatory challenge. These findings could help explain why obese humans show increased sensitivity to infections.

INTRODUCTION

Obesity is a very common disease that has reached epidemic status in many developing countries (Bessesen, 2008). It is defined by an excess accumulation of adipose tissue that causes significant health problems, such as cardiovascular disease and type II diabetes. Adipose tissue is a source of numerous inflammatory factors and thus obesity is associated with a change in inflammatory markers, including pro- and anti-inflammatory cytokines (Fantuzzi, 2005; Juge-Aubry et al., 2005). The consequence of this change in inflammatory state is not clear, but might be linked to the increase in susceptibility and morbidity to infections reported in obese individuals (Falagas and Kompoti, 2006). Obesity is associated with poorer wound healing and increased infection following surgical procedures (Vilar-Compte et al., 2000), higher rates of infection and mortality after burns (Gottschlich et al., 1993), and an increased risk of sepsis in the critically ill (Bercault et al., 2004; Yaegashi et al., 2005; Vachharajani, 2008). Furthermore, higher rates of respiratory, periodontal and skin infections occur in the obese population (Al Zahri et al., 2003; Garcia, 2002; Sabato et al., 2006; Salerno et al., 2004; Thorsteinsdottir et al., 2005; Wood et al., 2003).

An altered immune response to infection has also been observed in several genetic animal models of obesity, including ob/ob and db/db mice, and the Zucker fa/fa rat (Faggioni et al., 1997; Faggioni et al., 1999; Ivanov et al., 2001; Ivanov and Romanovsky, 2002; Lugarini et al., 2005; Mancuso et al., 2002; Rosenthal et al., 1996; Ordway et al., 2008; Hsu et al., 2007; Ikejima et al., 2005; Park et al., 2009; Wehrens et al., 2008; O’Connor et al., 2005; Plotkin et al., 1996). These animals are obese owing to either a deficiency in the adipokine leptin (ob/ob) or a defective leptin receptor (db/db and fa/fa). In addition to its involvement in energy balance, recent evidence suggests that leptin also plays a role in the regulation of immunity (Lam and Lu, 2007). It is possible, therefore, that the effects observed in genetically obese animals are due to a lack of leptin or leptin signalling rather than the effects of obesity per se. Furthermore, although genetically obese rodents have proven to be useful models of obesity, genetic mutations leading to leptin deficiency or a defective leptin receptor have been identified only within a small subset of the human population (Farooqi, 2008). Therefore diet-induced obesity (DIO) in rodents is a more physiologically relevant model of human obesity because the majority of cases of obesity in humans is associated with positive energy balance (increased food intake with or without decreased energy expenditure).

To date, few studies have examined how DIO rodents respond to bacterial or viral infection (Amar et al., 2007; Smith et al., 2007; Strandberg et al., 2009), and only one study in rats has investigated how DIO affects the behavioural and inflammatory response to Gram-negative bacterial endotoxin (Pohl et al., 2009). Endotoxin is the most important microbial trigger in the life-threatening condition sepsis (systemic inflammation due to infection), which kills 4 million people a year worldwide (Cohen, 2002). Most patients with sepsis develop fever; however, in more severe cases, patients develop hypothermia that is often associated with a worse outcome (Aron et al., 1999; Clemmer et al., 1992). In rodents, gram-negative bacterial infections are associated with several host defence responses, such as sickness behaviour, which is an adaptive response to infection characterised by altered sleep patterns, a loss of appetite and body...
weight, changes in body temperature, and social withdrawal. Lipopolysaccharide (LPS) is the active component of endotoxin from Gram-negative bacteria and causes sickness behaviour in animals, including anorexia and changes in body temperature (fever or hypothermia), effects that are mediated by the central actions of cytokines such as interleukin-1 beta (IL-1$\beta$) and IL-6.

The aim of this study was to determine the behavioural consequences of LPS administration in DIO mice by assessing food intake, body weight and core body temperature. In order to identify possible mechanisms underlying the effect of DIO on systemic inflammation, we determined the cytokine profile [IL-1 receptor antagonist (IL-1RA), IL-1$\beta$ and IL-6] in response to LPS in peripheral tissues, macrophages and blood. Finally, because the actions of LPS on behaviour are mediated by the brain, we assessed the effect of LPS on neuronal activity in DIO mice by quantifying Fos protein expression in key brain regions. We also compared the effects of LPS in DIO mice to ob/ob mice.

RESULTS

Experiment 1: effect of LPS (100 $\mu$g/kg) on food intake, body weight and core body temperature in DIO mice

After 20 weeks maintenance on a high-fat diet, DIO mice weighed significantly more than control mice (control, 33.7±1.1 g vs DIO, 52.4±1.1 g; P<0.001). Intraperitoneal (i.p.) administration of 100 $\mu$g LPS/kg body weight significantly reduced food intake and body weight in control mice at 8 hours and at 1 day after injection (Fig. 1A,B). The effect of LPS in control mice was transient: there was no difference in food intake or body weight between vehicle- or LPS-treated control mice at 2-4 days. By contrast, LPS had a greater and more prolonged effect in DIO mice. LPS caused a significant reduction in food intake and body weight in DIO mice at 8 hours compared with vehicle-treated DIO mice, and these effects were still observed 3 (body weight) and 4 (food intake) days after injection (Fig. 1A,C). The reduction in food intake and body weight in DIO mice was significantly greater at days 1-4 and 1-3, respectively, compared with control mice (P<0.05).

Injection of 100 $\mu$g LPS/kg body weight in control mice caused a significant rise in core body temperature that lasted until approximately 8 hours after injection (Fig. 1D,F). By contrast, LPS did not induce a febrile response in DIO mice. (F) Analysis of the change in core body temperature over 0-8 hours after injection is illustrated as the area under the curve (AUC; °C.h). Data are mean ± s.e.m. for n=8-10 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs respective vehicle-treated animals; $P<0.05$ vs LPS-treated control animals.

Experiment 2: effect of LPS (100 $\mu$g/kg) on food intake, body weight and core body temperature in ob/ob mice

Prior to injection, obese ob/ob mice weighed significantly more than lean controls (control, 26.9±0.3 g vs ob/ob, 41.9±0.6 g; P<0.001). In control mice, i.p. injection of LPS (100 $\mu$g/kg) caused a transient reduction in food intake and body weight at 1 day after injection.
However, the effect of LPS in obese ob/ob mice was observed earlier, with LPS inducing a decrease in food intake and body weight at 8 hours after injection. Body weight was also reduced in ob/ob mice at 1 day post-LPS injection, in the absence of a significant effect on food intake (Fig. 2A,C).

Injection of LPS (100 μg/kg) had no significant effect on core body temperature in control mice over the 8-hour monitoring period (Fig. 2D,F). However, a significant increase in core body temperature was observed in ob/ob after LPS injection (Fig. 2E,F).

Experiment 3: effect of LPS (5 μg) on food intake, body weight and core body temperature in DIO mice
After 20 weeks on a high-fat diet, DIO mice weighed significantly more than mice fed a standard control diet (control, 33.0±0.6 g vs DIO, 47.6±1.3 g; P<0.001). As with 100 μg LPS/kg body weight, 5 μg LPS in control mice produced a transient reduction in food intake at 8 hours and at 1 day after injection, and a decrease in body weight at 1 day after injection (Fig. 3A,B). By contrast, 5 μg LPS had a prolonged effect in DIO mice; in these mice, a decrease in food intake and body weight was observed between 8 hours and 2-3 days after injection (Fig. 3A,C), and the reduction in body weight was greater in LPS-treated DIO mice compared with control mice at 8 hours and at 2 days (P<0.05; Fig. 3B,C).

L.P. injection of 5 μg LPS in control mice had no significant effect on core body temperature during the first 8 hours after injection (Fig. 3D,F). However, LPS induced an increase in core body temperature during this time in DIO mice (Fig. 3E,F).

Experiment 4: effect of LPS (5 μg) on food intake, body weight and core body temperature in ob/ob mice
Prior to injection, obese ob/ob mice weighed significantly more than lean controls (control, 31.6±0.3 g vs ob/ob, 49.1±0.8 g; P<0.001). In control mice, LPS (5 μg, i.p.) caused a significant decrease in food intake between 1 and 3 days after injection (Fig. 4A). Compared with control mice, an anorexic effect of LPS was observed earlier in ob/ob mice: a reduction in food intake was noted at 8 hours and lasted until 3 days after injection (Fig. 4A). A reduction in body weight in response to LPS was observed in control mice at 8 hours and at 1 day after injection (Fig. 4B), an effect that lasted until 2 days in obese ob/ob mice (Fig. 4C).

LPS (5 μg, i.p.) caused a significant increase in core body temperature over 8 hours in both control and ob/ob mice (Fig. 4D-F). However, the effect of LPS on temperature in ob/ob mice was greater, with the rise in body temperature in these mice being significantly higher compared with LPS-treated controls (Fig. 4F).

Experiment 5: effect of LPS on Fos expression in the brain and on peripheral cytokine release in DIO mice
After 20 weeks maintenance on their respective diets, DIO mice were significantly heavier than control mice (body weight: control, 35.3±1.4 g vs DIO, 45.0±1.0 g; P<0.001). At 2 hours after i.p. administration of LPS (100 μg/kg) to control mice, a significant increase in Fos expression was observed in several forebrain regions, including the bed nucleus of stria terminalis (BNST), subfornical organ (SFO), supraoptic nucleus of the hypothalamus (SON), paraventricular...
nucleus of the hypothalamus (PVN), amygdala, and the nucleus of the tractus solarius (NTS) and area postrema (AP) of the brainstem when compared with vehicle-treated control mice (Fig. 5A,B). In LPS-treated DIO mice, a significant increase in the number of Fos-positive cells was observed in the same brain regions reported for control mice, apart from the SFO and SON, where no significant change in Fos expression was detected compared with vehicle-treated DIO mice. However, the number of cells positive for Fos protein in the PVN, NTS and AP was significantly lower in LPS-treated DIO mice compared with LPS-treated controls.

At 2 hours after i.p. administration of 100 μg LPS/kg body weight, there was a significant increase in IL-6 and IL-1RA levels in the plasma of control and DIO mice compared with their respective vehicle-treated groups (P<0.001; Table 1). There was no significant difference between plasma levels of IL-6 or IL-1RA after LPS treatment in control versus DIO mice. The amount of IL-1β detected in the plasma 2 hours after LPS in control or DIO mice was below the level of detection. LPS caused a significant increase in IL-1β protein in the liver of control mice (P<0.001) but failed to stimulate expression in DIO mice. LPS had no effect on the production of IL-6 or IL-1RA in the liver 2 hours after injection in DIO or control mice.

Peripheral administration of LPS stimulated the expression of IL-1β and IL-6 to the same extent in the spleen of control compared with DIO mice. LPS also significantly increased IL-1RA expression in the spleen of control mice, but had no effect on IL-1RA expression in the spleen of DIO mice.

Experiment 6: effect of LPS on Fos expression in the brain and on peripheral cytokine release in ob/ob mice

At the time of injection, ob/ob mice were significantly heavier compared with control mice (body weight: control, 24.1±0.3 g vs ob/ob, 44.5±1.0 g; P<0.001). At 2 hours after i.p. administration of LPS (100 μg/kg) to control mice, a significant increase in Fos expression was observed in several forebrain regions, including the BNST, SFO, SON, PVN, amygdala, and the NTS and AP of the brainstem when compared with vehicle-treated control mice (Fig. 5C). In ob/ob mice, LPS caused a significant increase in the number of Fos-positive cells only in the SFO, PVN and AP; no significant change was detected in the BNST, SON, amygdala and NTS when compared with vehicle-treated ob/ob mice. In addition, the number of cells positive for Fos protein in the SON and NTS was significantly higher in vehicle-treated ob/ob compared with control mice.

Peripheral injection of LPS (100 μg/kg) had no effect on the plasma levels of IL-1β in control mice but significantly increased IL-1β in ob/ob mice 2 hours after injection (Table 1). LPS significantly increased the levels of IL-6 and IL-1RA in the plasma of both control and ob/ob mice when compared with vehicle-treated mice. However, there was less IL-6 but more IL-1RA in the plasma of LPS-treated ob/ob mice compared with LPS-treated controls. LPS significantly increased IL-1β to the same extent in the liver of both control and ob/ob mice, but had no effect on the levels of IL-6 or IL-1RA in either group of mice. At 2 hours after injection,
LPS increased IL-1β and IL-6 protein in the spleen of both control and ob/ob mice. LPS also stimulated the expression of IL-1RA in the spleen of control mice, but had no effect in ob/ob mice.

**Experiments 7 and 8: effect of LPS on the release of cytokines from peritoneal macrophages in DIO and ob/ob mice**

LPS significantly increased peritoneal macrophage IL-6 content and release to the same degree in cells from control or DIO mice (Table 2). LPS also caused a significant increase in IL-1β expression, to an equivalent level, in macrophages isolated from either control or DIO mice (P<0.01 and P<0.05, respectively). ATP-induced release of IL-1β was significantly increased in the supernatant of cells from LPS-treated control and DIO mice compared with their respective vehicle-treated groups. However, there was significantly less IL-1β released in response to LPS and ATP in macrophages from DIO compared with control mice. Consistent with this, reduced IL-1β release was also observed in the supernatants of LPS-treated macrophages from ob/ob mice compared with macrophages isolated from control mice (data not shown). LPS had no effect on the amount of IL-1RA detected in the supernatant or cell lysate of peritoneal macrophages from either control or DIO mice.

**DISCUSSION**

This study demonstrates, for the first time, that DIO mice exhibit an altered anorexic, temperature and inflammatory response to bacterial endotoxin (LPS) compared with control mice, and suggests that obesity might impair the ability of the immune system to appropriately respond to bacterial infection. Furthermore, we also report for the first time that LPS causes a greater febrile response in obese ob/ob mice than in controls.

The anorexic response to peripheral administration of LPS in DIO mice was enhanced and prolonged compared with control mice: DIO animals showed a greater reduction in food intake and body weight, and took longer to recover after LPS treatment. Although previous studies have demonstrated that DIO rats display a greater febrile response to LPS (Pohl et al., 2009), and genetically obese leptin-deficient ob/ob mice are more sensitive to the anorexic actions of LPS (Faggioni et al., 1997), the present study is the first to report on the anorexic and febrile effects of LPS in DIO and ob/ob mice. Because only a small number of obese humans are deficient in leptin (Farooqi, 2008), DIO is arguably a more relevant model of human obesity and, therefore, our data in DIO mice could have relevance to the increase in susceptibility and morbidity to infection reported in obese individuals (Falagas and Kompoti, 2006).

In the present study, the initial dose of LPS administered was based on body weight (i.e. 100 μg LPS/kg body weight). Therefore, it is likely that, owing to their increased body weight, obese mice would have elevated levels of peritoneal LPS that could be responsible for the heightened anorexic response observed. In order to address this issue, we assessed the anorexic (and febrile) effects of a fixed dose of LPS (i.e. 5 μg) regardless of body weight, and observed that DIO and ob/ob mice again displayed an altered response to LPS.
response to LPS compared with controls. These data suggest, therefore, that obese mice are highly sensitive to the anorexic actions of LPS.

The present study also demonstrates that DIO and ob/ob mice exhibit an altered temperature response to LPS. In some experiments, LPS did not induce a rise in core body temperature (fever) in control mice. In our hands it is difficult to induce a consistent febrile response in mice at the doses of LPS used here because these effects can also be influenced by slight fluctuations in ambient temperature. However, both DIO and ob/ob mice showed a greater rise in core body temperature to a dose of 5 μg LPS, compared with controls, and only ob/ob mice displayed an enhanced febrile response to 100 μg/kg LPS: this latter dose of LPS did not induce a fever in DIO mice. However, interpretation of these data is complicated because, in some DIO mice, LPS induced hypothermia (data not shown). The thermoregulatory response to LPS in rodents is complex and depends on the ambient temperature and dose administered (Rudaya et al., 2005; Wang et al., 1997; Romanovsky et al., 2005). The ambient temperature range in the present study (19-24°C) was sub-neutral and, at these temperatures, LPS induces a fever at low doses, mild hypothermia followed by fever at intermediary doses, and prolonged hypothermia at high doses that induce septic shock (Rudaya et al., 2005). Because LPS (at 100 μg/kg) induced hypothermia in some DIO mice in the present study, it is possible that these mice are more sensitive to the thermoregulatory effects of LPS.

The mechanism underlying the altered thermoregulatory response to LPS in DIO and ob/ob mice in the present study is yet to be determined. Previous studies have demonstrated that the obese Koletsky rat (which lacks the leptin receptor) exhibits a prolonged hypothermia in response to high doses of LPS (Steiner et al., 2004), which, among other things, could be due to impaired brown adipose tissue (BAT) thermogenesis (Steiner and Romanovsky, 2007). However, although it remains to be determined whether DIO and ob/ob mice have a reduced capacity to increase BAT thermogenesis after infection, our data do suggest that DIO mice are more likely than control mice to develop septic shock in response to systemic inflammation, because the incidence of LPS-induced hypothermia was greater in DIO mice compared with controls. Hypothermia in mice correlates with mortality after bacterial infection (Vlach et al., 2000) and, in support, mice fed a high-fat/cholesterol diet show enhanced mortality to a high dose of LPS.

Fig. 5. Effect of i.p. injection of LPS on Fos protein expression in the brains of control, DIO and ob/ob mice.

(A) Representative photomicrographs illustrating Fos expression in the brain of control (a-d and i-l) or DIO (e-h and m-p) mice after i.p. injection of vehicle (5 ml saline/kg body weight) or LPS (100 μg/kg body weight). After LPS injection, significant increases in Fos expression were observed in the SFO (a,b,e,f) and SON (c,d,g,h) in control mice only, and in the PVN (j,m,n) and AP (k,l,o,p) of control and DIO mice, although the number of Fos-positive cells in response to LPS was lower in DIO compared with control mice. Scale bars: 100 μm. 3V, third ventricle; cc, central canal. (B) Quantification of the number of Fos-positive nuclei per section in DIO mice. (C) Quantification of the number of Fos-positive nuclei per section in ob/ob mice. No photomicrographs are shown for ob/ob mice because findings were similar to DIO mice. Data are mean ± s.e.m. for n=5 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs respective vehicle-treated group; #P<0.05, ##P<0.01 versus LPS-treated control; $P<0.05, $$P<0.01 versus vehicle-treated control.
Table 1. Cytokine expression in the plasma, liver and spleen in response to LPS in DIO and ob/ob mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DIO</th>
<th>ob/ob mice experiment</th>
<th>Control</th>
<th>DIO</th>
<th>Vehicle</th>
<th>LPS</th>
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<tr>
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<td></td>
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<tr>
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<td>&lt;31</td>
<td>&lt;63</td>
<td>&lt;63</td>
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<tr>
<td>IL-6 (ng/ml)</td>
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<td>21±6</td>
<td>&lt;0.03</td>
<td>21±4</td>
<td>&lt;0.03</td>
<td>14±2</td>
<td>&lt;0.03</td>
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<td>IL-1RA (ng/ml)</td>
<td>0.3±0.2</td>
<td>8±1</td>
<td>0.7±0.1</td>
<td>12±2</td>
<td>0.2±0.1</td>
<td>7±1*</td>
<td>0.8±0.2</td>
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<td><strong>Liver</strong></td>
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<tr>
<td>IL-1β (pg/mg protein)</td>
<td>188±82</td>
<td>996±197</td>
<td>523±135</td>
<td>413±136</td>
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<td>111±28</td>
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<td>3100±1300</td>
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<td>4900±1400</td>
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<td>1713±98</td>
<td>2498±265</td>
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<td><strong>Spleen</strong></td>
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Samples were taken 2 hours after LPS (100 µg/kg, i.p.) or vehicle (5 ml/kg saline) injection from DIO and ob/ob mice, and their respective controls. Cytokine expression was analysed by ELISA. Data are mean ± s.e.m. for 5 mice per group. 
P<0.05 versus LPS-treated control mice. 
P<0.01 versus respective LPS-treated groups (and LPS-treated groups for IL-1β expression to the same degree in obese and control macrophages. Overall, this attenuated cytokine release in DIO and ob/ob mice might seem unexpected because these mice show greater behavioural responses to LPS. However, a blunted, or delayed, peripheral cytokine response is also associated with an increase in mortality and morbidity in DIO mice after bacterial Porphyromonas gingivalis or influenza virus infection (Amar et al., 2007; Smith et al., 2007), or in ob/ob mice in response to Listeria monocytogenes or LPS (Ikejima et al., 2005; Faggioni et al., 1999). Furthermore, DIO mice showed a delayed recovery in response to LPS and, thus, the impairment in the release of IL-1 and IL-1RA suggests that DIO mice might have a defect in mounting an appropriate immune response, resulting in inadequate elimination of bacterial infection.

In agreement with the present study, a reduction in IL-1RA expression in response to LPS is also observed in obese db/db (O’Connor et al., 2005) and ob/ob (Faggioni et al., 1999) mice in the spleen and plasma, respectively. As demonstrated here and by others, these genetically obese mice also display increased sensitivity to the actions of LPS (O’Connor et al., 2005; Faggioni et al., 1997; Faggioni et al., 1999). Mice deficient in IL-1RA show

Table 2. Cytokine expression and release from cultured peritoneal macrophages from DIO mice after LPS injection

<table>
<thead>
<tr>
<th></th>
<th>IL-1β (pg/ml)</th>
<th>IL-1RA (ng/ml)</th>
<th>IL-6 (pg/ml)</th>
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<tr>
<td></td>
<td>Control</td>
<td>DIO</td>
<td>Control</td>
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<td>&lt;63</td>
<td>2±4</td>
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<tr>
<td>LPS</td>
<td>&lt;63</td>
<td>&lt;63</td>
<td>4±8</td>
</tr>
<tr>
<td>LPS + ATP</td>
<td>1871±575</td>
<td>1099±362</td>
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<td>Vehicle</td>
<td>&lt;63</td>
<td>&lt;63</td>
<td>20±1</td>
</tr>
<tr>
<td>LPS</td>
<td>2070±531</td>
<td>1696±687</td>
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Cytokine expression and release in cell lysates and supernatants, respectively, were analysed by ELISA in peritoneal macrophages from DIO and control mice. Cytokine expression was induced by LPS (1 µg/ml, 2 hours). LPS does not induce secretion of IL-1β, but subsequent challenge with ATP induces release of IL-1β into the culture supernatant. Data are mean ± s.e.m. for cultures prepared from n=4-5 mice per group. 
P<0.05 versus respective vehicle-treated groups (and LPS-treated groups for IL-1β experiment); 
P<0.05 versus LPS + ATP-treated control mice.
an increase in endotoxin-induced lethality (Hirsch et al., 1996), and endogenous IL-1RA limits LPS-induced fever (Cartmell et al., 2001); thus, it is possible that the lack of IL-1RA release from the spleen in response to LPS is responsible for the enhanced sickness behaviour in DIO and ob/ob mice reported here. However, because the present study only assessed cytokine expression at 2 hours after LPS, a more detailed time course is required in order to assess the temporal profile of the inflammatory response to infection in DIO and ob/ob mice.

The mechanism underlying the altered systemic immune response in DIO and ob/ob mice reported here and by others (Amar et al., 2007; Smith et al., 2007; Faggioni et al., 1999; Ikejima et al., 2005) is unclear. In the present study, peritoneal macrophages and immune cells within the spleen and liver of DIO and ob/ob mice showed a reduced capacity to release the pro- and anti-inflammator

METHODS

Animals and diets

C57BL/6 male mice (8 weeks old; Harlan UK Limited, UK) were randomly assigned to either a high-fat diet (60% fat by energy; Test Diets, supplied by IPS Product Supplies, UK) or a standard diet (12% fat by energy; Test Diets, supplied by IPS Product Supplies) for 20 weeks. Obese ob/ob mice (10-12 weeks of age; Harlan UK Limited, UK) and normal weight controls (10-12 weeks of age; Harlan UK Limited) were maintained on a standard laboratory chow diet (10% fat by energy; Beekay International, UK). Mice on their respective diets were group housed and body weight and food intake of mice were measured weekly. All mice were given ad libitum access to food and water, and were housed at a constant ambient temperature of 21±2°C on a 12-hour light, 12-hour dark cycle (lights on at 08:00 h). All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986. Animals fed a high-fat diet are referred to as DIO mice, and mice fed a control diet as controls.

Experiments 1-4: the effect of LPS on food intake, body weight and core body temperature in DIO and ob/ob mice

DIO and control mice after 20 weeks of maintenance on their respective diets, or ob/ob and control mice at 10-11 weeks of age, were anaesthetised with isoflurane (1.5-2.5% in O2) and radiotransmitters (TA10TA-F20; Data Sciences, MN) were implanted abdominally into the peritoneum to allow for measurement of core body temperature by remote radiotelemetry. After surgery, mice were allowed to recover for 7-9 days before being housed individually 24 hours before experimental injections. Mice (n=5-10 per group) were then injected intraperitoneally (i.p., at 10:00 h) with either LPS (0127:B8 from Escherichia coli; Sigma-Aldrich, UK) or vehicle (sterile saline at 5 ml/kg body weight). After injections, animals were given a pre-weighed amount of their respective diets, and core body temperature was measured continuously in undisturbed animals by radiotelemetry.

In the first experiment, LPS was administered at a dose of 100 μg/kg (based on body weight). However, because DIO and ob/ob mice received a larger total amount of LPS, owing to their greater body weight compared with control mice, separate experiments were performed using a fixed dose of LPS (5 μg). Food intake and body weight were monitored daily for 4 days and core body temperature was monitored for 8 hours after injection. Experiments involving DIO or ob/ob mice were performed as separate studies.
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Experiments 5 and 6: effect of LPS on Fos expression in the brain and peripheral release of cytokines in DIO and ob/ob mice

Separate groups of DIO (high-fat fed for 20 weeks) or ob/ob mice (10 weeks of age) and their respective controls (n=5 per group) were handled daily for 7 days (to reduce stress), after which they were given an i.p. injection of either LPS (100 μg/kg) or vehicle (5 ml/kg sterile saline). At 2 hours post-injection, animals were anaesthetised with sodium pentobarbitone (i.p.; Sagatal, Rhône-Mérieux, UK), blood was sampled by cardiac puncture and plasma obtained after centrifugation (13,000 g, 10 minutes). Mice were then perfused transcardially with 0.9% heparinised saline, and samples of liver and spleen taken. Plasma, liver and spleen samples were frozen and stored at –80°C until analysis by ELISA. Following perfusion with saline, animals were then perfuse-fixed with 4% paraformaldehyde [in 0.1 M phosphate buffer (PB)]. Brains were removed, post-fixed overnight in the same fixative, and cryopreserved in 30% sucrose. Coronal 30 μm brain sections were cut throughout the level of the hypothalamus and brainstem on a freezing slide microtome. Immunohistochemistry for Fos protein was then performed on free-floating sections. Endogenous peroxidase was removed before treatment in blocking solution (2% normal goat serum in PB/0.3% Triton X-100). Sections were then incubated at 4°C overnight with a rabbit polyclonal anti-Fos antibody (1:5000, Ab5; Merck Chemicals, UK), washed in PB/0.3% Triton X-100 and treated for 2 hours in a peroxidase-labelled goat anti-rabbit IgG antibody (1:500; Vector Laboratories, UK). Following washes (in 0.1 M PB), nuclear Fos was detected by incubation in a nickel-diaminobenzidine solution (Sigma-Aldrich) that produced a blue-black precipitate.

The brain regions analysed for Fos expression were those previously reported to express Fos after i.p. injection of LPS (Sagar et al., 1995). The number of immunopositive cells expressing Fos protein per section (2-11 sections depending on the region analysed) was counted bilaterally, using a light microscope, in nuclei defined by Franklin and Paxinos (Franklin and Paxinos, 1997): BNST, 0.38 to 0.14 mm; SFO, 0.02 to –0.82 mm; SON, –0.58 to –0.94 mm; PVN, –0.58 to –1.22 mm; central nucleus of the amygdala, –0.70 to –1.82 mm; NTS, –6.34 to –7.48 mm; AP, –7.20 to –7.48 mm. The average number of cells per section was calculated and the group mean determined for each brain region.

Experiments 7 and 8: effect of LPS on cytokine release from peritoneal macrophages in DIO and ob/ob mice

Macrophages were prepared as described previously (Le Feuvre et al., 2002) from DIO or control mice after 20 weeks on their respective diets (n=4-5 per group) or from ob/ob or control mice (n=5-6 per group) at 12 weeks of age. Briefly, following sacrifice, the peritoneal cavity was lavaged with 8 ml RPMI 1640 containing 5% fetal bovine serum, 100 mg/ml streptomycin and 100 IU penicillin (all from Invitrogen, UK). The medium was recovered and cells were collected by centrifugation (80 g, 5 minutes) and seeded onto a 24-well plate at a density of 1×10⁶ cells/ml; the macrophages were then incubated overnight (37°C, 5% CO₂). To induce the synthesis of pro-IL-1β, IL-6 and IL-1RA, the macrophages were incubated with 1 mg/ml LPS (026:B6, E. coli; Sigma-Aldrich) for 2 hours. A further stimulation of the macrophages with ATP (5 mM, 10 minutes) to activate the P2X7 receptor and induce the release of IL-1β into the culture supernatant (Le Feuvre et al., 2002) was performed to investigate the effects of obesity on the capability of the macrophages to secrete IL-1β. Cell supernatant and lysate were then collected and stored at –20°C until analysis by ELISA.

Cytokine measurement: ELISA

Saline-perfused liver and spleen samples (from experiments 5 and 6) were homogenised in buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂ and 0.02% Na₂HPO₄) containing 1% Triton X-100 and a protease inhibitor cocktail (Set I; Calbiochem, Merck Chemicals). All homogenates were centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant from the liver samples was further ultracentrifuged at 100,000 g for 1 hour at 4°C. Supernatant for both spleen and liver were then stored at –20°C until analysis. Mouse IL-1β, IL-6 or IL-1RA concentrations in plasma, homogenates from liver and spleen, and supernatant and cell lysates from macrophages (from experiments 7 and 8) were analysed by ELISA (Duosets; R&D Systems, UK) according to the manufacturer’s instructions. Cytokine concentrations were determined using the manufacturer’s instructions.
determined by reference to the relevant standard curves. For liver and spleen supernatant, protein concentration was assessed by a BCA protein assay (Pierce Biotechnology, Rockford, IL) and results expressed as pg/mg protein.

Data analyses and statistics
All data are presented as mean ± s.e.m. Cumulative food intake is calculated as kcal and expressed as % of control, because the control and high-fat diets have different metabolisable energy contents based on weight (g). Body weight and body temperatures are plotted as the mean change from the point of injection (time zero). For body temperature, the integrated temperature response between 0 and 8 hours [area under the curve (AUC), °C·h] was calculated for each animal by the trapezoidal method. To take into account the animals that exhibited a hypothermic response, the baseline was set at 0 and peaks both above and below the baseline were considered. The AUC for the positive and negative peaks was determined and the net AUC calculated. Average net AUC values for each animal by the trapezoidal method. To take into account body temperature, the integrated temperature response between

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
C.B.L. conceived and designed the experiments, analysed the data and wrote the paper. C.B.L. performed the majority of the in vivo experiments and ex vivo analyses, with the help of E.M.K., and D.B. performed the experiments involving peritoneal macrophages.

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