Reduced stress- and cold-induced increase in energy expenditure in interleukin-6-deficient mice

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Wernstedt, Ingrid, Amanda Edgley, Anna Berndtsson, Jenny Fält, Göran Bergström, Ville Wallenius, and John-Olov Jansson. Reduced stress- and cold-induced increase in energy expenditure in interleukin-6-deficient mice. Am J Physiol Regul Integr Comp Physiol 291: R551–R557, 2006. —Interleukin-6 (IL-6) deficient (-/-) mice develop mature onset obesity. Pharmacological studies have shown that IL-6 has direct lipolytic effects and when administered centrally increases sympathetic outflow. However, the metabolic functions of endogenous IL-6 are not fully elucidated. We aimed to investigate the effect of IL-6 deficiency with respect to cold exposure and cage-switch stress, that is, situations that normally increase sympathetic outflow. Energy metabolism, core temperature, heart rate, and activity were investigated in young preobese IL-6-/- mice by indirect calorimetry together with telemetry. Baseline measurements and the effect of cage-switch stress were investigated at thermoneutrality (30°C) and at room temperature (20°C). The effect of cold exposure was investigated at 4°C. At 30°C, the basal core temperature was 0.6 ± 0.24°C lower in IL-6-/- mice compared with wild-type mice, whereas the oxygen consumption did not differ significantly. The respiratory exchange ratio at 20°C was significantly higher and the calculated fat utilization rate was lower in IL-6-/- compared with wild-type mice, suggesting a lower cold-induced thermogenesis in IL-6-/- mice. In addition, SNS activity is negatively correlated with RER (42). On the other hand, the SNS stimulation has been reported to increase production and release of IL-6 from peripheral tissues (32). It has even been reported that sympathetic neurons can both respond to and produce IL-6 themselves (31). Diverse models of stress increase production and release IL-6 into the circulation (44, 45). Moreover, treatment with β-adrenergic receptor agonists induces IL-6 release from fat tissue (32). Infusion of IL-6 in both rats and humans has been shown to stimulate lipolysis, fat utilization, and energy expenditure, indicating that peripheral IL-6 exerts antiobesity effects, at least transiently (34, 44, 47, 52).

Thus it is unknown how endogenous IL-6 suppresses body fat, but there is growing evidence that central IL-6 increases sympathetic outflow. This could be a possible mechanism for the antiobesity effect of IL-6, as low SNS activity, low energy expenditure, and high respiratory exchange ratio (RER) have all been identified as predictors of weight gain (30, 37, 42). In addition, SNS activity is negatively correlated with RER (42). To elucidate the possible interactions between endogenous IL-6 and the SNS, we have compared the responses of wild type and IL-6-/- mice during conditions with known increases in sympathetic outflow, such as new-cage stress and cold exposure. We have also measured norepinephrine in plasma, as well as the response to injected norepinephrine to investigate possible differences in the SNS regulation between wild-type and IL-6-/- mice.

INTERLEUKIN-6-DEFICIENT (IL-6-/-) mice display increased body weight and body fat mass late in life (10, 13, 50) without significant changes in food intake in relation to body weight (50). Pharmacological treatment of intact rats with intracerebroventricular IL-6 causes increased energy expenditure and decreased body fat, whereas the same dose administered intraperitoneally had no effect (40, 49). Moreover, adenoassociated viral vector expressing murine IL-6 directly delivered into the rat hypothalamus enhances uncoupling protein-1 (UCP-1) protein levels in brown adipose tissue (BAT), via a sympathetic nervous system (SNS)-dependent mechanism (27).

IL-6 seems to be of importance for the regulation of body fat also in humans. Cerebrospinal fluid levels of IL-6 in male subjects were negatively correlated to different indices of obesity but did not correlate to the IL-6 levels in the circulation (43). Furthermore, a variant of the human IL-6 gene promoter that leads to reduced IL-6 production has been associated with increased body fat mass and decreased energy expenditure in several studies (4, 19, 24, 54). In addition, in the IL-6 receptor-α, one microsatellite CA repeat (11) and one single nucleotide polymorphism (358 Asp→Ala) (55) have been associated with obesity.

There are several interactions between IL-6 and the SNS. As mentioned above, central delivery of IL-6 may stimulate the SNS (27). On the other hand, the SNS stimulation has been reported to increase production and release of IL-6 from peripheral tissues (32). It has even been reported that sympathetic neurons can both respond to and produce IL-6 themselves (31). Diverse models of stress increase production and release IL-6 into the circulation (44, 45). Moreover, treatment with β-adrenergic receptor agonists induces IL-6 release from fat tissue (32). Infusion of IL-6 in both rats and humans has been shown to stimulate lipolysis, fat utilization, and energy expenditure, indicating that peripheral IL-6 exerts antiobesity effects, at least transiently (34, 44, 47, 52).

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METHODS

Animals. The IL-6−/− mice were originally generated by Kopf et al. (22) and have been bred onto a C57BL/6 background [as described by Wallenius (50)] to reduce genetic heterogeneity. Littermate wild-type mice were used as controls in all experiments. Animals were maintained in separate cages under standardized nonbarrier conditions and had free access to fresh water and food pellets (B&K Universal AB, Sollentuna, Sweden).

All animal procedures were approved by the local ethics committee on animal care at Göteborg University and were conducted in accordance to guidelines.

Telemetry and oxygen consumption analyses. Telemetry devices (PDT-4000 HR E-Mitter, weight 2.2 g, MiniMitter, Sunriver, OR) were implanted in 5-mo-old IL-6−/− mice and littermate controls according to the surgery protocol provided by the manufacturer. In general, at this age, wild-type and IL-6−/− mice from our colony do not differ in body weight (50). To improve recovery, the mice were given subcutaneous injections of ~1 ml saline and 5 mg/kg Rofem-enVen (Veter AB, Södertälje, Sweden). The mice were left to recover from surgery for 2–3 wk before experiments were commenced. By this time, all mice had regained their preoperation body weights and were continuing to increase in weight. Food intake was recorded throughout the study.

Oxygen consumption and carbon dioxide production was measured by indirect calorimetry with a SOMEDIC Metabolic system, INCA (Somedic, Hörgård, Sweden). The SOMEDIC system is built around a sealed chamber, ventilated with a constant flow of air and regulated to a preset temperature range. The indirect calorimeter is also combined with a Mini-Mitter telemetry system, which is designed to measure body core temperature, three-dimensional locomotor activity and heart rate using implanted PDT-4000 HR E-Mitter. Data from the PDT-4000 HR E-Mitter is synchronized with and integrated in an INCA output data file. The body temperature is measured with a resolution of 0.01°C, the heart rate is measured with a resolution of one beat per minute (bpm), and the activity is determined as the number of changes in direction during 1 min. Oxygen consumption data were collected every 2 min, whereas all other variables were collected every minute. Lipid and carbohydrate utilization rate was calculated by the formula adapted from Consolazio et al. (8). Mice were conscious and unrestrained during the measuring periods. The same mice were not examined more often than once a week.

Baseline measurements. Baseline measurements were performed at 30°C and at room temperature (20°C) during an 8-h period (9 AM to 5 PM). Baseline body temperature, heart rate, and oxygen consumption was estimated by calculating the average of data obtained in periods where no locomotor activity occurred (when the value registered by the telemetry system was 0). Examinations of body fat and lean body mass by dual X-ray absorptiometry are, for technical and practical reasons, not feasible after implantation of telemetry device. In this study, oxygen consumption is expressed in milliliters per hour per milligram body weight. The tissue samples were dissected from anesthetized 2.5-mo-old male wild-type and IL-6−/− mice. The tissue samples were anesthetized with 60 mg/kg pentobarbital sodium to avoid uncontrolled stress responses before intraperitoneal injection of 1 mg/kg norepinephrine. Pentobarbital sodium does not interfere with norepinephrine responses (35). Oxygen consumption and core body temperature were measured 30 min before and 60 min after the norepinephrine injection, and the response was calculated by subtracting the highest achieved value with the lowest basal value. The measurement was performed at 32°C to keep the mice warm throughout the procedure.

UCP-1 protein measurements. Intrascapular BAT was dissected from anesthetized 2.5-mo-old male wild-type and IL-6−/− mice. The tissues were immediately frozen in N2 (l) and the stored in −80°C until analysis. The tissues were homogenized in 0.8 ml lysis buffer (10 mM EDTA, 0.25 M sucrose, 0.1% Triton X-100 and 0.05 mM deoxycholic acid) containing 50 μl/ml protease inhibitor cocktail (1 tablet of Complete, Roche, Germany) and were centrifuged at 10,000 g for 10 min at 4°C. The protein concentration was measured just before the electrophoresis by the RC/DC Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg), pre-treated with 4× SDS buffer, were separated on gel (NuPAGE 10% Novex Bis-Tris gel; Invitrogen, Lidingö, Sweden) with a NuPage MOPS SDS running buffer (Invitrogen) under reducing conditions. The separated samples were electrophoretically transferred to polyvi-
nyliden difluoride membranes (Amersham International, Little Chalfont, Buckinghamshire, UK) and treated with blocking buffer (0.2% I-Block, 0.2% BSA, 5 mM MgCl₂, 3 mM NaF, and 0.5% Tween-20 in PBS pH 7.4) for 4 h. The membrane was incubated with rabbit anti-UCP-1 antibody (Abcam, Cambridge, UK) at 1:4,000 dilution in blocking buffer overnight at 4°C. After washing in blocking buffer for 2 h, the membrane was incubated with alkaline phosphatase-linked polyclonal secondary antibody (Amersham International, UK) at 1:40,000 dilution in blocking buffer for 2 h. The detection was performed with CDP-Star substrate for alkaline phosphatase (Tropix, Bedford, MA). Immunoblotted signals were exposed and developed using enhanced chemiluminescence film (Amersham International) and subsequently scanned into a computer. Bands were quantified by densitometry using Scion Image software (Beta 4.02, Scion, Frederick, MD). After the UCP-1 analysis, the membrane incubated in stripping solution (62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol, pH 6.8) for 30 min at 60°C. The membrane was then washed and blocked and immunoblotted for GAPDH (1:5,000 dilution in blocking buffer overnight at 4°C. After washing in blocking buffer for 2 h, the membrane was incubated with alkaline phosphatase-linked polyclonal secondary antibody (Amersham International, UK) using the same procedure as described above. The UCP-1 protein expression was normalized for the GAPDH protein expression.

**Statistics.** All analyses were performed using a SPSS program (version 11.5.1; SPSS, Chicago, IL). In general, the nonparametric Mann-Whitney U-test was used. However, two-way repeated-measures ANOVA was used to compare variables that were measured repeatedly in the same animal. Moreover, two-way ANOVA was used to elucidate whether other factors beside genotype affected the results. Bivariate correlations were done using Pearson’s correlation coefficient. Data are presented as means ± SE, and P values < 0.05 were considered significant. The statistical method that was used is indicated in connection with the respective result.

**RESULTS**

**Baseline measurements.** In line with the results of an earlier report (13), the baseline RER at room temperature (20°C) was significantly higher in IL-6/−/− mice than in wild-type animals (0.79 ± 0.01 vs. 0.83 ± 0.01 for wild-type and IL-6/−/− mice, respectively) (Fig. 1A). Moreover, calculation of fat and carbohydrate utilization rate by means of a previously described method (8) showed that the fat utilization rate was significantly lower in IL-6/−/− compared with wild-type mice (Fig. 1B). At thermoneutrality, the RER was equal in wild-type and IL-6/−/− mice (0.85 ± 0.02 vs. 0.86 ± 0.02 for wild-type and IL-6/−/− mice, respectively). There was a trend for decreased baseline oxygen consumption in IL-6/−/− mice at thermoneutrality (0.89 ± 0.05 and 0.83 ± 0.02 ml/min for wild-type and IL-6/−/− mice, respectively), whereas the oxygen consumption at 20°C was equal in wild-type and IL-6/−/− mice (1.73 ± 0.12 and 1.72 ± 0.08 ml/min for wild-type and IL-6/−/− mice, respectively). Moreover, the core body temperature at thermoneutrality was significantly lower in IL-6/−/− mice (35.43 ± 0.15°C vs. 34.86 ± 0.18°C for wild-type and IL-6/−/− mice, P = 0.029). There was also a tendency for decreased body temperature in IL-6/−/− mice at room temperature (0.93 ± 0.53°C lower, P = 0.095). Heart rate did not differ between wild-type and IL-6/−/− mice at 30°C (354 ± 16.9 vs. 341 ± 10 beats/min, respectively) or at 20°C (499 ± 16.8 vs. 532 ± 18.4 beats/min, respectively). Furthermore, locomotor activity did not differ between wild-type and IL-6/−/− mice (data not shown). The mice were matched for body weight at 20°C but not at 30°C. Body weight was positively correlated with oxygen consumption (R = 0.66, P < 0.01), but not with core temperature (R = 0.24, P = 0.33) at 30°C. Moreover, two-way ANOVA analysis suggested that body weight is not of importance for core temperature in these mice (data not shown).

**Cage-switch stress test.** IL-6/−/− mice responded significantly less to new-cage stress compared with wild-type mice. Both at thermoneutrality (30°C) and at room temperature (20°C), the increase in oxygen consumption was less pronounced in IL-6/−/− mice (Fig. 2, A and B). The increase in heart rate in response to cage-switch stress was lower in IL-6/−/− mice than in wild-type mice at 30°C, but not at 20°C (Fig. 2, C and D). Despite the lower oxygen consumption and heart rate in IL-6/−/− mice, the level of locomotor activity was equal in the two groups (data not shown).

Fifteen minutes after a cage switch, the corticosterone levels increased approximately twofold (P = 0.007 for wild-type and P = 0.002 for IL-6/−/− mice). The cage-switch-induced corticosterone levels did not differ significantly between IL-6/−/− (201.7 ± 15.2 ng/ml; n = 9) and wild-type (139.3 ± 20.2 ng/ml; n = 6) mice. Likewise, corticosterone levels at baseline did not differ between IL-6/−/− (73.7 ± 5.8 ng/ml) and wild-type (68.3 ± 7.6 ng/ml) mice.

Plasma norepinephrine levels in female and male mice after cage-switch and anesthesia were slightly lower in IL-6/−/− compared with wild-type mice (Fig. 3). However, anesthetized male and female IL-6/−/− mice responded in the same way as wild-type mice to norepinephrine treatment (Table 1).

**Cold-induced thermogenesis.** At 4°C, IL-6/−/− mice had compared with wild-type mice significantly decreased oxygen consumption at baseline (Fig. 4A). This indicates that IL-6/−/− mice have decreased capacity for cold-induced thermogenesis. In line with this, the IL-6/−/− mice had about 2.5°C lower core temperature than wild-type mice (Fig. 4B), which is considerably larger than the observed temperature differences at 30°C.
and 20°C (see Baseline measurements). As the ability to defend core temperature is dependent on the degree of insulation and the body surface/volume ratio of the animal, we accounted body weight for the analysis. The body weight adjusted core temperature for wild-type and IL-6-/-- mice was 34.6 ± 0.66 and 30.8 ± 0.74°C, respectively (P<0.008 for the effect of genotype). The weight-adjusted oxygen consumption was 3.89 ± 0.08 and 3.48 ± 0.09 ml/min, in wild-type and IL-6-/-- mice, respectively (P<0.02 for the effect of genotype). Two-way ANOVA analyses of the data indicated that the genotype was the strongest determinant of core temperature and oxygen consumption in this experiment. There was no difference between IL-6-/-- and wild-type mice in heart rate and locomotor activity at 4°C. Moreover, the protein levels of UCP-1 in BAT did not differ between wild-type and IL-6-/-- mice (1.29 ± 0.17 and 1.29 ± 0.18 arbitrary units UCP-1/GAPDH for wild-type and IL-6-/-- mice, P=0.98).

**DISCUSSION**

The results of the present study show that lack of endogenous IL-6 is associated with a blunted increase in energy expenditure during exposure to new-cage stress and cold ambient temperature in young IL-6-/-- mice. The IL-6-/-- mice also had lower body temperature than the wild-type mice, especially during cold exposure. In addition, the IL-6-/-- mice had decreased fat utilization during baseline conditions at room temperature. Moreover, the increase in heart rate after cage-switch test at thermoneutrality was blunted in IL-6-/-- mice. Taken together, our data suggest that energy expenditure and thermogenesis are decreased in mice lacking endogenous IL-6. This effect may contribute to the previously reported (10, 13, 50) development of mature onset obesity in IL-6-/-- mice.

**Table 1. Response to norepinephrine treatment in anesthetized male and female wild-type and IL-6-/-- mice**

<table>
<thead>
<tr>
<th>Gender</th>
<th>NE Response</th>
<th>WT</th>
<th>IL-6-/--</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>ΔVO2, ml/min</td>
<td>1.50±0.13</td>
<td>1.52±0.17</td>
</tr>
<tr>
<td></td>
<td>ΔCore T, °C</td>
<td>3.62±0.69</td>
<td>3.36±0.55</td>
</tr>
<tr>
<td>Female</td>
<td>ΔVO2, ml/min</td>
<td>0.84±0.06</td>
<td>0.89±0.11</td>
</tr>
<tr>
<td></td>
<td>ΔCore T, °C</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Males: wild-type (WT), n = 5, IL-6-/--, n = 8; females: WT, n = 5, IL-6-/--, n = 5). Statistical comparisons were done with Mann-Whitney U-test. ND, not determined. NE, norepinephrine.
The present findings indicate that the increase in energy expenditure in response to stress and cold ambient temperature are blunted in IL-6−/− mice in the absence of effect on motor activity. Although a subtle effect on motor activity is difficult to completely rule out, these findings are in line with a role for endogenous IL-6 in stimulation of the SNS in mice. This assumption is supported by the blunted stress-induced increase in heart rate that we observed in IL-6−/− mice. The results of several previous studies have also indicated that IL-6 treatment can stimulate the SNS (27, 31). On the other hand, the results of other studies are inconsistent with an effect of IL-6 deficiency on SNS activity (25). Moreover, our own data provide only weak support for an effect of IL-6 deficiency on basal SNS activity at thermoneutrality, or on SNS activity during mild cold-induced stress at 20°C.

A possible mediator of central effects of IL-6 on SNS is CRF in the paraventricular nucleus (PVN) of the hypothalamus. CRF has been reported to stimulate the SNS and the PVN is known to have neuronal links to the brain stem and to contribute to regulation of SNS activity (21, 39). Moreover, there are indications that IL-6 is a key stimulator of hypothalamic CRF production during pathophysiological conditions such as inflammation (7, 51). It remains to be investigated whether IL-6 has a role in regulation of CRF during health. The effect of IL-6 on sympathetic outflow at the level of CNS could be exerted by locally produced IL-6 rather than IL-6 transported over the blood-brain barrier from the circulation. Unlike for leptin, there is no correlation between levels of IL-6 in cerebrospinal fluid and blood, and the levels of IL-6 in cerebrospinal fluid are often higher than those in blood (43).

We found decreased norepinephrine levels in IL-6−/− mice, although recently published data indicated increased norepinephrine levels in IL-6−/− mice (25). However, measurements of catecholamines in plasma are highly dependent on sampling technique and conditions (15, 17), and this might explain the discrepancy. In any case, the low, norepinephrine levels observed in IL-6−/− mice in the present study are consistent with results from a previous study (13). When controlling for other factors, a low lipid-to-carbohydrate oxidation, which is an inheritable trait (57), is an established risk factor for weight gain in humans (41, 57) and may also contribute to development of obesity later in life in IL-6−/− mice. The mechanism behind decreased lipid utilization in IL-6−/− mice is not clear. It could be due to an effect of IL-6 at the level of the CNS causing decreased sympathetic outflow in these mice. It has been shown that hypothalamic stimulation can affect respiratory quotient (RQ; 2), and high RQ has been associated with sympathetic activity (42). However, the fact that decreased lipolysis was also seen during basal condition (room temperature at rest), that is, when the heart rate was equal in wild-type and IL-6−/− mice, may argue against a general difference in SNS activity. Alternatively, there is evidence that IL-6 may exert lipolytic and fat-burning effects also when given outside the CNS, effects that may not involve the SNS. Several studies have shown that infusion of IL-6 to healthy human subjects increases fatty acid oxidation and/or turnover (28, 36, 52). A possible mechanism for this peripheral effect.
could be that IL-6 increases the concentration and activity of the AMP kinase-activated protein kinase (20), which exerts similar effects on metabolism as epinephrine and the SNS (5).

Moreover, peripheral IL-6 treatment has been reported to decrease LPL activity, which may cause net lipolysis (16).

We found lower stress-induced increment in heart rate in IL-6−/− than in wild-type mice at thermoneutrality (30°C) but not at room temperature (20°C). The latter finding is in line with a recently published study that found no effect of IL-6 deficiency on cage-switch-induced increment in heart rate at room temperature (25). The reason for a difference between room temperature and thermoneutrality is unknown. However, increased sympathetic and decreased vagal control of heart rate even in the absence of stress at room temperature might interfere with stress-induced increase in heart rate, as tachycardia often is associated with both increased SNS and vagal withdrawal (9).

The lack of difference in baseline and stress-stimulated corticosterone secretion levels between IL-6−/− and wild-type mice suggests that IL-6 is not crucial for activation of the HPA-axis. This is in line with another study showing a normal corticosterone response after stress in IL-6−/− mice (29). In addition, central IL-6 delivery did not alter corticosterone levels in rats (27). However, more studies are needed to evaluate the role of endogenous IL-6 for glucocorticoid production. One possibility is that, although IL-6 modulates the HPA-axis during inflammatory states (48, 53), it may not be crucial for corticosterone release during psychological stress.

The findings of increased obesity and decreased energy expenditure and thermogenesis in the IL-6−/− mice may be relevant in a human context. This view is supported by studies on IL-6 gene polymorphisms in human populations. We and others have found that the C allele of the common single nucleotide −174 G/C polymorphism in the IL-6 gene promoter, which has lower IL-6 transcription rate (3, 14, 38, 46, 56), is associated with overweight (4, 19, 54). This weaker IL-6 promoter variant is also associated with lower energy expenditure (24) and a slightly lower body temperature (1).

In conclusion, the present results indicate that IL-6 deficiency in mice decreases the increments in oxygen consumption in response to moderate stress and cold exposure and lowers core body temperature. Moreover, IL-6 deficiency seems to lower lipid utilization at room temperature. A decrease in energy expenditure and lipid oxidation in young mice could contribute to the previously reported development of mature onset obesity. Results from clinical studies indicate that genetically determined differences in IL-6 production may change energy balance, thermogenesis, and fat mass accumulation also in humans. In the long run, small imbalances in the energy balance can result in cumulative increments in fat mass and eventually obesity.

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DISCLOSURES

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