Summation of behavioral and immunological stress: metabolic consequences to the growing mouse

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Laugero, Kevin D., and Gary P. Moberg. Summation of behavioral and immunological stress: metabolic consequences to the growing mouse. Am J Physiol Endocrinol Metab 279: E44–E49, 2000.—To address the hypothesis that multiple stressors can have cumulative effects on the individual, we determined the effects of restraint (R) stress (4 h/day for 7 days), immunological (L) stress [lipopolysaccharide (LPS) injection, 0.45 μg/g body wt on days 6 and 7], and R + L (RL) on the growth and energetics of C57Bl/6 male mice. R and L each repeatedly increased (P < 0.05) circulating corticosterone (>8 times), but RL caused even greater (>250%; P < 0.05) concentrations of circulating corticosterone than did either stressor alone. Only L and RL increased (P < 0.05) circulating interleukin-1β. Although R, L, and RL impaired growth (>75% below controls, P < 0.05), RL reduced growth to a greater extent. All stressors inhibited (P < 0.05) lean (>33% below controls) and fat (>120% below controls) energy deposition, and like the effects on growth, combined RL stress inhibited lean and fat energy deposition to a greater extent than did either stressor acting alone. These results demonstrated that the summation of multiple stress results in a cumulative cost to the growing animal.

stress summation; corticosterone; interleukin-1; energy partitioning; growth

ALL STRESS COMES AT A biological cost to the individual (28, 29). For most stressors, this cost is insignificant because of their brief duration. However, whereas a single or brief episode of stress may not amount to a significant biological cost, repeated or multiple stress may summate and impose a cost to normal biological function or lead to the development of pathology. We recently found (25) that growing mice are at greater risk of losing normal biological function (e.g., growth) when they are repeatedly exposed to acute behavioral stress. The accumulation of repeated exposure to this acute stress caused a significant disruption of growth, which resulted in the failure of these mice to reach normal body weight compared with age-matched controls. However, the depression of body weight, which occurred over the first few days of stress, plateaued to a level that was maintained by the repeated stress over the remaining experimental period. This led us to question whether addition of exposure to immunological stress would summate with the repeated behavioral stress to further depress growth and body weight, leading to a cumulative cost that was greater than the cost of either stress by itself.

Although this concept of stress summation has been proposed (6, 26, 28) or implied (20, 35), it had not been experimentally tested. To address this hypothesis, we examined the biological cost of combined exposure to repeated behavioral stress and immunological stress. Because the stress-induced shift of energy from normal biological function might account for the impairment of energy-sensitive functions such as growth (6, 12, 34), we quantified the effect of these multiple stressors on the growth (change in body weight) and energetics of growing mice. To confirm the induction of behavioral stress, we analyzed circulating corticosterone; to confirm the induction of immunological stress, we determined circulating interleukin-1β (IL-1β).

MATERIALS AND METHODS

Male C57Bl/6 mice (B & K Universal, Fremont, CA) were individually housed in hanging wire-mesh cages in a room maintained at 23 ± 2°C with lights on for 14 h (0700 to 2100) per day. Before experimentation, mice were acclimated for 1 wk, and all experiments began when the mice were 31 days old. Each day at 0700, body weight was recorded, feed was removed, and feed intake (corrected for spillage) was measured. All mice were then returned to their home cages. During the 4-h restraint period (0700–1100), treated groups and controls did not have access to food and water. Immediately after the 4-h restraint period, all mice were fed a semipurified test diet (PMI AIN-76A, PMI Feeds, St. Louis, MO) having a guaranteed analysis of 18.4% protein, 5.0% fat, 65.0% carbohydrate, and 5.0% fiber. With the exception of the 4-h restraint period, all mice had ad libitum access to feed and water. All experiments were approved by the University of California Davis Animal Care and Use Committee.

Experiment 1: Effect of multiple stressors on growth and energetics. Experiment 1 lasted 7 days, beginning at 0700 on day 1 and ending at 0700 on day 8. On day 1, all mice were weighed and exposed to one of four treatments: behavioral stress (R), immunological stress (L), behavioral stress coupled with immunological stress (RL), or no stress (Con). For behavioral stress, mice were placed daily for 7 days into a
well ventilated restraint tube, as previously described (18). Each episode of restraint lasted 4 h. For immunological stress, a lipopolysaccharide (LPS) was injected into the peri-
toneal cavity (27, 37). LPS (from Escherichia coli O55:B5, Sigma, St. Louis, MO) was injected twice daily at 0.45 µg/g body wt on days 6 and 7 of the experiment. Mice in the L group were treated like Con on days 1–5 and were injected with LPS on days 6 and 7. Mice receiving the RL treatment were injected with LPS and restrained immediately there-
after.

Energy measurements. A comparative slaughter exper-
imental design was employed to quantify the 7-day lean and fat tissue energy changes (2). In each experiment that exam-
ined energy changes, an initial group of mice (31 days old) was decapitated and dissected into eviscerated carcass (C), gastrointestinal tract (contents removed) plus liver (GI/
Liver), and remaining viscera (V). These components were
analyzed for water (freeze-drying to constant weight), fat (difference in weight of dried component before and after ether-acetone extraction), and protein (Kjeldahl N × 6.25) content. Carcass lean energy (CLE), GI/Liver lean energy (GLE), and viscera lean energy (VLE) were determined by multiplying the dry gram content by the energy content of protein (assumed to be 5.4 kcal/g). Carcass fat energy (CFE), GI/Liver fat energy (GFE), and viscera fat energy (VFE) were determined by multiplying the dry gram content by the energy content of fat (assumed to be 9.0 kcal/g). Regression equations were generated from these data to express total and carcass FE and LE as linear functions of body weight at age 31 days (Table 1).

On the final day of experiment 1, experimental mice were decapitated, dissected, and analyzed as described for 31-day-
old mice. Changes in C, GI/Liver, and V water, fat energy (FEΔ), and lean energy (LEΔ) were determined by the differ-
ence between the final (measured) and the initial (predicted
from regression equations) values. Total body change in lean and fat energy content was taken as the sum of C, GI/Liver,
and V energy changes. Change in total body energy (BEΔ) was taken as the sum of LEΔ + FEΔ.

The 7-day metabolizable energy intake (MEI) was calcul-
ated as the gram intake multiplied by the metabolizable
energy content of the diet (3.79 kcal/g at maintenance; PMI
Feeds). For mice gaining protein over the 7-day study, 1.4
cal/kg of protein gain was added to the value of total MEI (2). Heat energy (HE) was estimated by taking the difference
between MEI and BEΔ. Gross energetic efficiency was calcu-
lated by dividing the BEΔ by the MEI (BEΔ/MEI). Experi-
ment 1 was replicated, and because the effects of stress on
growth did not differ between replicates, data were pooled.

Experiment 2: Effect of multiple stressors on corticosterone, IL-1β, and insulin-like growth factor I. To obtain sufficient
blood for hormone analyses and to avoid the increase in
corticosterone associated with the stress of blood collection, it
was necessary to kill the mice (within 30 s of removal from
their home cage) by decapitation for the collection of trunk
blood. Therefore, in parallel with experiment 1, experiment 2 examined the effect of multiple stressors over the 7-day
experimental period on the dynamics of circulating cortico-
sterone, insulin-like growth factor I (IGF-I), and IL-1β. In
experiment 2, mice were randomly assigned to one of the four
treatments detailed under Experiment 1. Blood was collected 4 h after the initiation of restraint on days 1, 3, 6, and 7. At
the selected times, a predetermined group of mice was decap-
itated, and trunk blood was collected into heparinized (15
U/ml blood) tubes kept on ice and subsequently centrifuged
at 1,000 g for 30 min. After centrifugation, plasma was
collected and stored at −70°C until analyzed for corticoste-
rone, IL-1β, and IGF-I.

Hormone/cytokine assays. Plasma corticosterone concen-
trations were determined by RIA with the use of a corti-
osterone-125I system (ICN, Costa Mesa, CA). Samples
were run in duplicate, and the intra- and interassay coef-
ficients of variation were determined as 3.1 and 8.1%,
respectively. Plasma IGF-I was extracted by means of an
acid-ethanol procedure with cryoprecipitation (5). Human
recombinant IGF-I (R & D Systems, Minneapolis, MN) was
iodinated and purified as described (19). IGF-I concentra-
tions were determined by a nonequilibrium RIA (5). The
polyclonal IGF-I antiserum (UB3–189), kindly provided by
Drs. J. J. Van Wyk and L. Underwood, was obtained
through the National Institute of Diabetes and Digestive
and Kidney Diseases National Hormone and Pituitary
Program. Samples were run in duplicate, and the intra-
and interassay coefficients of variation were determined
as 4.2 and 8.2%, respectively. Plasma IL-1β concentrations
were determined by a quantitative sandwich enzyme immu-
noassay (R & D Systems). Samples were run in dupli-
cate, and the intra- and interassay coefficients of variation
were determined as 3.2 and 7.6%, respectively.

Statistical analyses. All data were analyzed by least-
squares analysis of variance procedures (SAS/STAT User’s
initial and final body weights and the total MEI, body
weight gain, LEΔ, FEΔ, energetic efficiency (BEΔ/MEI),
and HE over 7 days were analyzed in a statistical model
that included the effect of treatment. For hormone data,
differences between treatment groups were analyzed in a
model that included the effects of treatment, the day, and
their interaction. Where indicated, as a means of adjusting
energetic responses to a common MEI, analysis of covari-
ce was employed. For all analyses, differences in means
were determined with the PDIFF option in PROC GLM
(SAS/STAT User’s Guide), and a level of P < 0.10 was
considered statistically significant. A level of P < 0.10 was
considered to indicate tendencies.

Table 1. Linear regression equations from experiment 1*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression Equation</th>
<th>r²</th>
</tr>
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<tbody>
<tr>
<td>TLE</td>
<td>0.89 (±0.05) body wt, - 1.729 (±0.86)</td>
<td>0.97</td>
</tr>
<tr>
<td>TFE</td>
<td>1.22 (±0.15) body wt, - 3.397 (±2.49)</td>
<td>0.83</td>
</tr>
<tr>
<td>CLE</td>
<td>0.71 (±0.03) body wt, - 0.864 (±0.63)</td>
<td>0.96</td>
</tr>
<tr>
<td>CFE</td>
<td>1.13 (±0.14) body wt, - 4.047 (±2.34)</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Equations express indicated parameter as a function of initial
body weight (body wt) and were used to predict initial (day 1) energy
content in experimental mice. TLE, total body lean energy; TFE, total
body fat energy; CLE, carcass lean energy; CFE, carcass fat
energy. For each parameter, n = 13 mice and P = 0.0001.
did either stressor alone. Although body weights did not differ before the experiment (day 1), repeated restraint, LPS injection, and repeated restraint coupled with LPS injection reduced \( P < 0.0001 \) final body weight (day 8); however, the multiple stress of repeated restraint plus LPS injection reduced final body weight to a greater extent than did either repeated restraint \( P < 0.0001 \) or LPS injection \( P < 0.0003 \) alone.

Actual HE was reduced by each of the stressors (Fig. 2C). However, because HE production depends on MEI (3, 4, 11, 23), which was affected by stress, and because behavioral and immunological stress can increase basal HE production (1, 16, 31), we also assessed HE production after adjusting for the differences in MEI between treatment groups. When the stress-induced differences in MEI were removed through the use of covariate analysis, HE was significantly increased \( P < 0.0001 \) by all three stressors and to a greater extent by the summation of behavioral and immunological stress \( P < 0.0001 \).

The gross energetic efficiency was analyzed to determine the effects of stress on energy deposition, independent of any effect of MEI. All stressors significantly reduced \( P < 0.0001 \) gross energetic efficiency, but the multiple stressor depressed it to a greater degree \( P < 0.0001 \) than did either stressor alone. Mice exposed to the summation of the two stressors lost \( 0.190 \pm 0.011 \) kcal body energy per unit MEI compared with mice exposed to behavioral stress or immunological stress.
which lost 0.030 ± 0.011 and 0.020 ± 0.011 kcal body energy per unit MEI, respectively.

Experiment 2: Hormonal and cytokine response to multiple stressors. In general, circulating corticosterone and IGF-I concentrations were significantly affected by stress ($P < 0.0001$). Although the mean levels of these hormones were not constant over the experiment ($P_{\text{day}} < 0.0067$), the effect of stress treatment on the circulating concentrations of corticosterone and IGF-I were not dependent on the day ($P_{\text{stress} \times \text{day}} > 0.10$; Fig. 3). Although restraint and LPS injection each increased corticosterone ($P < 0.0001$), exposure to the combination of the two stressors increased circulating levels of this hormone to a greater extent than did exposure to either restraint ($P < 0.0204$) or LPS injection ($P < 0.0006$). All three stressors depressed circulating IGF-I to a similar degree compared with non-stressed controls, and there were no differences between mice exposed to either repeated restraint, LPS injection, or the combination of these stressors. LPS and day affected ($P < 0.0060$) circulating IL-1β, and a significant ($P < 0.0300$) stress $\times$ day interaction was present (Fig. 3C). Only mice injected with LPS (L and RL) had elevated IL-1β ($P < 0.0001$). Compared with control and repeatedly restrained mice, circulating IL-1β was significantly higher in mice exposed to LPS and the combination of restraint plus LPS on both days of LPS injection. The increase in IL-1β induced by the multiple stressor was significantly ($P < 0.0470$) greater on day 6 than on day 7, and the circulating cytokine did not differ between mice exposed to LPS or restraint plus LPS on either day of LPS injection.

**DISCUSSION**

In the present work, we found that, when the effects of a single stressor repeated daily for 7 days are combined with the effects of an immunological stressor, the total biological cost was greater than the cost of either stressor by itself. Although behavioral stress is known to inhibit growth ($7, 17, 30$), we demonstrated here that animals undergoing behavioral stress were even more prone to growth inhibition when faced with an additional stressor. Each episode of restraint was perceived as stressful to the animal, as indicated by increased circulating corticosterone (Fig. 3A). LPS injection induced immunological stress, as indicated by increased corticosterone and IL-1β. Behavioral stress and immunological stress each suppressed normal growth, whereas combined exposure to behavioral and immunological stress had a greater impact on growth than either stressor alone (Fig. 1).

At least part of the behavioral and immunological stress-induced growth impairment observed in this study can be explained by altered energy deposition into both lean and fat tissues. Simultaneous exposure to both stressors impaired energy deposition to a greater extent than either stressor by itself, which may account for the larger inhibitory effect of combined stress exposure on energy deposition. Both behavioral and immunological stress can depress energy intake ($10, 22, 24$), and our measure of MEI is in agreement with those reports. We demonstrated that animals experiencing multiple stressors suffered an even greater inhibition of MEI, which may explain why these animals had greater growth impairment than mice experiencing the smaller depressive effects on MEI of either behavioral or immunological stress alone. However, the gross energetic efficiency was also more greatly reduced by multiple stressors than by either behavioral or immunological stress alone. Because gross energetic efficiency estimates the capacity of the animal to deposit energy into tissues from an equivalent unit of energy intake, these results suggest that some additional stress-related alteration(s), independent of the effect of depressed intake, was responsible for the enhanced impairment of energy deposition in mice experiencing multiple stressors.
We believe that one alteration responsible for the greater suppressive effects of the multiple stressors is increased basal heat energy production. Although it has been reported that basal heat energy production is increased during behavioral and immunological stress (1, 11, 16, 31, 36, 37), restraint, LPS injection, and restraint coupled with LPS injection decreased total heat energy in the present study. However, heat energy depends on MEI (3, 4, 11, 23), which was altered by these stressors. Further examination showed that, when the effects of MEI were removed, all three stressors significantly increased heat energy, and the combination of behavioral and immunological stress increased heat energy to a greater degree than did either stressor alone. These results suggest that basal metabolic heat production was increased by all three stressors and to a greater extent by the multiple stressors. Thus mice exposed to multiple stressors probably had an overall heat energy that was higher than one would have expected in mice (R or L) with an equivalent reduction of MEI. Therefore, compared with the restraint and LPS treatments, the greater elevation of basal heat energy elicited by the multiple stressors was a cost that increased the quantity of energy partitioned into heat as opposed to growth and may have accounted in part for the impaired growth observed in mice exposed to both behavioral and immunological stress.

Immunological stress results in an increase of circulating cytokines (e.g., IL-1β and tumor necrosis factor-α), which have a wide-ranging effect on metabolism (32). After LPS injection or an infectious challenge, these cytokines increase energy expenditure and lean and fat tissue catabolism and cause anorexia (1, 15, 22, 32). In the present study, LPS administration induced immunological stress in both restrained and nonrestrained mice, as indicated by increased circulating IL-1β and corticosterone. Additionally, the multiple stressors caused greater increases in increased circulating corticosterone than did either restraint or LPS injection on day 6 and 7. Because elevated circulating corticosterone, as seen in the present study, is known to have catabolic effects on both protein and fat tissue (21) and to inhibit growth and caloric efficiency (8, 9, 33), exposure to higher circulating concentrations of corticosterone may explain in part why behaviorally stressed mice incurred even greater depression in growth and energy deposition when additionally exposed to immunological stress. Furthermore, the additional effects of increased circulating cytokines, such as IL-1 on energy deposition, may have summated with the effects of corticosterone to bring about greater impairment of energy deposition and growth in mice exposed to repeated behavioral stress plus immunological stress.

Our results in the present study support the hypothesis that individuals experiencing stress are at greater risk of losing normal biological function when they are faced with an additional stressor. It will be important in the future to define the individual contributions of each stressor to the total biological cost and to determine the impact that any one stressor may have on this cost.

We thank Dr. Chris Calvert for expert advice on growth and energetics and for guidance in many areas of the experimentation. Our thanks go to Dr. Anita M. Oberbauer for help with the IGF-I assay, and we are also grateful to Dr. Edward J. DePeters and Scott Taylor for technical help with nutritional analyses.

REFERENCES


