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Hypermetabolic Syndrome as a Consequence of Repeated Psychological Stress in Mice

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Stress is a powerful modulator of neuroendocrine, behavioral, and immunological functions. After 4.5-d repeated combined acoustic and restraint stress as a murine model of chronic psychological stress, severe metabolic dysregulations became detectable in female BALB/c mice. Stress-induced alterations of metabolic processes that were found in a hepatic mRNA expression profiling were verified by *in vivo* analyses. Repeatedly stressed mice developed a hypermetabolic syndrome with the severe loss of lean body mass, hyperglycemia, dyslipidemia, increased amino acid turnover, and acidosis. This was associated with hypercortisolism, hyperleptinemia, insulin resistance, and hypothyroidism. In contrast, after a single acute stress exposure, changes in expression of metabolic genes were much less pronounced and predominantly confined to gluconeogenesis, probably indicating that metabolic disturbances might be initiated already early but will only manifest in repeatedly stressed mice. Thus, in our murine model, repeated stress caused severe metabolic dysregulations, leading to a drastic reduction of the individual's energy reserves. Under such circumstances stress may further reduce the ability to cope with new stressors such as infection or cancer. (*Endocrinology* 149: 2714–2723, 2008)

CATABOLIC PROCESSES for energy mobilization and anabolic functions such as growth or healing need to be balanced to sustain health (1–5). Psychological and physiological stressors can disturb neuroendocrine, immunological, behavioral, and metabolic functions (1, 4, 6–8), and adaptive physiological processes aim to reconstitute a dynamic equilibrium (1, 9). These stress responses are normally short lasting and physiologically important for survival to cope with a changing environment or to deal with potentially life-threatening situations (1, 9). However, in modern society individuals are continuously confronted with stressful stimuli, and prolonged neuroendocrine responses probably harm rather than protect (1, 3, 10, 11). Stress-induced neuroendocrine alterations include activation of the sympathetic nervous system with increased secretion of catecholamines, and stimulation of the hypothalamus-pituitary-adrenal (HPA) axis with heightened release of glucocorticoids (GCs) (1, 8, 9). Prolonged and increased release of catecholamines is associated with cardiovascular diseases such as hypertension, myocardial infarction, or stroke (1, 5, 12). Excessive secretion of GCs was linked to diabetes, dyslipidemia, cardiovascular alterations, immunosuppression, and mood disorders (1, 5, 13).

Recently, we showed in a murine model of psychological stress that BALB/c mice develop severe systemic immunosuppression due to 4.5-d intermittent stress exposure that we rated as chronic stress.

This immunodeficient state was associated with lymphocytopenia, T-cell anergy, high rates of lymphocyte apoptosis in lymphoid organs, impaired phagocytic and oxidative burst responses, and induction of an antiinflammatory cytokine bias. On the one hand, stress-induced immunosuppression was accompanied with attenuation of a hyperinflammatory septic shock, but on the other hand, with a reduced clearance of experimental infections in the long term (8, 14). Furthermore, we documented behavioral alterations with increased depression-like behavior and neuroendocrine alterations such as prolonged activation of the HPA axis and increased turnover of catecholamines. Finally, a prominent stress-induced loss of body mass without significant changes of food and water intake during the observation period became detectable (8). It is known that stress exposure is linked to changes of body weight (BW). There is evidence that hypothalamic control of food intake is influenced by stress, which in consequence alters metabolism. In such a situation, some people lose and others gain weight in response. However, the molecular mechanisms of the stress-BW connection remain to be elucidated.

Several authors propose chronic stress to be a main feature in the pathogenesis of the metabolic syndrome, which is associated with obesity, type 2 diabetes/insulin resistance, dyslipidemia, and hypertension (3, 5, 7). On the other hand, stressors like injury, infection, traumatic events, or prolonged sleep deprivation capably induce hypercatabolism and, therefore, may cause cachexia (2, 3, 10, 15–20). Such metabolic-driven wasting may result from pain, depression,

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Abbreviations: BW, Body weight; GC, glucocorticoid; Got1, glutamate oxaloacetate transaminase 1; HDL, high-density lipoprotein; HE, hematoxylin-eosin; HPA, hypothalamus-pituitary-adrenal; IPA, Ingenuity Pathway Analysis; LDL, low-density lipoprotein; PAS, periodic acid-Schiff; VLDL, very low-density lipoprotein.

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or anxiety, causing malabsorption and maldigestion or morphological and functional alterations of the gastrointestinal system (3, 16, 21–24), and is typically seen during repeated inflammatory processes or during sepsis (2, 15–17).

In the literature, two main pathways leading to massive loss of body mass are discussed: the response to starvation and the hypermetabolic response. Starvation is induced by inadequate calorie intake and causes the use of the body's own tissue (3, 16, 25). At first, carbohydrate stores are emptied for energy production. Secondarily, the body switches to usage of lipid and protein stores for gluconeogenesis. Finally, fatty acids are absorbed into the liver where ketone bodies are produced that, for example, neurons can use as an energy source to restore functional homeostasis. In a feedback loop, efferent neurons signal to the periphery so that gluconeogenesis is lowered, and protein breakdown is diminished that causes adaptation to starvation (3, 16). The supply for basal energy production then comes from the calories of adipose stores (3, 26).

In contrast, a hypermetabolic response that is often seen during critical illness is predominantly mediated by hormones and inflammatory mediators (2, 6, 7, 15–17, 27). As during the initial phase of starvation, gluconeogenesis is accelerated by usage of lipids and amino acids (6, 17, 27, 28). Protein breakdown is massively increased due to glucagon and GC effects, and can be accelerated by proinflammatory cytokines such as TNF or IL-1 (16, 29, 30). Amino acids, along with fatty acids and glycerol, are used for gluconeogenesis in the liver, causing hyperglycemia (6, 31). This process is mainly mediated by catecholamines and corticosteroids, and finally results in a rapid loss of lean body mass without sufficient metabolic adaptation to diminish tissue breakdown (2, 16, 18, 32).

To get further clues that metabolic alterations account for the pronounced loss of BW in our repeated stress model, we decided for a global screening of gene expression in the liver.

Based on documented changes of the expression profile of hepatic genes involved in carbohydrate, lipid, and amino acid metabolism, we started to characterize metabolic disturbances and found biological relevant alterations of glucose metabolism, dyslipidemia, and changes in amino acid turnover in repeatedly stressed BALB/c mice.

Materials and Methods

Animal experiments

Female BALB/c mice aged 6–8 wk were randomly grouped into the experimental and control groups starting at least 4 wk before being used in experiments. The group size in different experiments differed from six to 12 mice per cage. Animals stayed in their group until the end of the experiments and were not mixed up to avoid social stress. All animals were maintained with sterilized food (ssniff R-Z; ssniff Spezialdiäten GmbH, Soest, Germany) and tap water *ad libitum* for adaptation under minimal stress conditions. Influences of irregularities of the estrous cycles of unisexually grouped female mice (33) were not analyzed selectively and may cause higher SD values in the statistical analyses.

Animal rooms had a 12-h light, 12-h dark cycle and were maintained at a constant environment before the experiment. To avoid any additional effect, *e.g.* acoustic or olfactory effects, the handling of mice during the adaptation period and during the experiments was restricted to one investigator. All animal procedures were performed as approved by the Ethics Committee for Animal Care of Mecklenburg-Vorpommern, Germany.

Repeated stress model

Mice were exposed to combined acoustic and restraint stress on 4 successive days, for 2 h twice a day during the physiological recovery phase of rodents (0800–1000 and 1600–1800 h). On d 5 only one stress session was performed in the morning. For immobilization mice were placed in 50-ml conical centrifuge tubes with multiple ventilation holes without penning the tail. Acoustic stress was induced by a randomized ultrasound emission device between 19 and 25 kHz with 0–35 dB waves in attacks (patent no. 109977; SiXiS, Taipei, Taiwan), allowing the mice no adaptation to the stressor (8, 14). Between the stress sessions, mice stayed in their home cages, and had free access to food and tap water. Control mice were kept isolated from stressed animals during the 4.5-d stress exposure to avoid any acoustic or olfactory communication between the groups. Therefore, the nonstressed group stayed in the incubator where the animals were adapted. The stressed mice remained outside the incubator in the same animal laboratory during the whole period of the stress model. All successive experiments and analyses were performed starting at 1000 h after the ninth stress exposure. Different *in vivo* analyses were performed with six to 12 mice per group in at least two experiments according to the experimental protocol to ensure reproducibility. For array analysis two independent stress experiments were performed with nine mice per group (first experiment) and eight mice per group (second experiment).

Acute stress model

Mice were exposed to a single 2-h combined acoustic and restraint stress cycle in the morning (0800–1000 h). *In vivo* analyses were performed immediately after or 6 h after the stress session with six to nine mice per group. Two acute stress experiments for array analysis were composed of eight animals for each control group, and nine animals per stress group in the first and eight animals per stress group in the second experiment.

Organ harvesting for RNA preparation

Mice were killed by cervical dislocation and organs were removed immediately to avoid RNA degradation. For liver samples a small piece of tissue was immediately homogenized with a micropestle in 350 μl Buffer RLT/ 1% β-Mercaptoethanol (QIAGEN GmbH, Hilden, Germany/Sigma-Aldrich Chemie GmbH, München, Germany). The liver lysates were shock frozen in liquid nitrogen. All samples were stored at −70 C.

RNA preparation

Liver sample lysates were thawed and processed at room temperature for RNA preparation with a RNeasy Mini Kit (QIAGEN GmbH) according to the manufacturer's instructions. After ethanol precipitation the RNA was quantified spectrophotometrically, and its quality was verified using an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies Inc., Santa Clara, CA).

DNA array analysis

For each group pools containing equal amounts of RNA from each individual animal were prepared and used for subsequent microarray analysis. Five micrograms of pooled total RNA were used for the synthesis of double-stranded cDNA, and this solution then served as a template for an *in vitro*-transcription reaction using GeneChip Expression 3′ Amplification Reagents (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. After spin-column based cleanup, concentration and quality of cRNA were measured as described previously.

cRNA was fragmented, added to the hybridization cocktail, denatured, and hybridized with Affymetrix GeneChip Mouse Expression Arrays 430A/430A 2.0 according to the manufacturer's instructions. Washing, staining, and scanning were performed using Affymetrix GeneChip FluidicsStation and scanners according to standard protocols.

DNA array data analysis

The Affymetrix expression analysis was performed for the livers of repeatedly stressed and healthy control mice with technical duplicates

of two independent biological experimental series each. For the analysis of the effects of acute stress, array hybridizations were also performed of two independent biological experiments for both groups (control and acute stress). Affymetrix array image data generated with MAS 5.0 (repeated stress) were analyzed using the GeneChip Operating Software 1.2 (Affymetrix) with default values for parameter settings. For normalization, a scaling procedure with a target value of 150 was used. Image data of the acute stress experiment were directly analyzed in GeneChip Operating Software 1.4 with default settings and normalized by scaling to the target value 500. After data transfer to the GeneSpring software package (Agilent Technologies), genes displaying differential regulation in response to repeated and acute stress were identified based on the following criteria: 1) the signal of probe sets had to be present in the arrays at least in the control (for repressed genes) or in stressed mice (for induced genes) in both biological experiments, 2) the difference of mean signals between control and stressed mice had to equal or exceed 100, and 3) the fold change factors calculated from the signal values in each experimental replicate had to exceed a cutoff of more than or equal to 1.5 or less than or equal to −1.5 in both biological experiments.

Lists of probe sets displaying differential regulation in both acute and repeated stress, or specifically after acute or repeated stress were then uploaded as Excel spreadsheets (Microsoft Corp., Redmond, WA) into the Ingenuity Pathway Analysis (IPA) Version 5.5 (Ingenuity Systems, Inc., Redwood City, CA; http://www.ingenuity.com) and used for the interpretation of the array data in the context of already published knowledge. Biological functions were assigned to the networks based on the content of the Ingenuity Pathway Knowledge Base. Complete hepatic gene expression data of stressed and nonstressed mice are available at the NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database and are accessible through GEO Series accession no. GSE11126.

Real-time PCR

DNA was removed by DNase treatment, and subsequent to purification using a RNeasy Micro Kit (QIAGEN GmbH) and ethanol precipitation, concentration and quality of RNA samples were assayed as described previously. Validation of expression data by real-time PCR was separately performed for all individual RNA preparations (n = 9 plus n = 8 mice per group) of the two biological experiments with repeated stress exposure. For real-time PCR analysis, 1 μg RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit in the presence of SUPERase•In RNase inhibitor (Ambion/Applied Biosystems, Foster City, CA). Twenty nanograms of cDNA served as a template for real-time PCR using the following 20× TaqMan Gene Expression Assays (Applied Biosystems): Asl (Mm00467107_m1), Srebf1 (Mm00550338_m1), Pck1 (Mm00440636_m1), Gadd45b (Mm00435123_m1), Sds (Mm00455126_m1), and Actb (Mm00607939_s1). Differential regulation in repeatedly stressed and control mice was confirmed by comparing the ΔCt values (Ct value of the target gene − Ct value of the reference gene Actb in identical cDNA samples) of all control mice and repeatedly stressed mice with a Mann-Whitney *U* test, requiring a *P* value of less than or equal to 0.05.

Assessment of BW, food intake, and water consumption

Body mass was determined for stressed and control mice before the first and immediately after the ninth stress session. Average food and water intake per cage of stressed and nonstressed groups was monitored during these 4.5-d stress exposures, including spillage, by measuring weight of food pellets (g) and volume of drinking water (ml) for cages with nine equally aged mice.

Harvesting of blood and organ samples

Immediately after the single (acute stress) or in the repeated stress model after the ninth stress session, mice were anesthetized with 75 μg/g BW ketamine Curamed (CuraMED Pharma GmbH, Karlsruhe, Germany) and 16 μg/g BW Rompun (Bayer AG, Leverkusen, Germany) diluted in pyrogen-free 0.9% sodium chloride (Braun, Melsungen, Germany). Blood was harvested by retro-orbital puncture and collected in K2E-EDTA Vacutainer tubes (BD Vacutainer, Plymouth, UK). Plasma samples were either analyzed immediately or stored at −20 C until use. Samples of adrenal glands, liver, and spleen for histological staining were immediately put into liquid nitrogen and stored at −80 C until further analysis.

Measurement of plasma hormones

Plasma levels of corticosterone (OCTEIA Corticosterone enzyme immunoassay; Immunodiagnostic Systems Ltd., Boldon, UK), insulin (mouse insulin ELISA kit; Mercodia AB, Uppsala, Sweden), resistin (mouse Resistin ELISA; BioVendor, Munich, Germany), GH (Diagnostic Systems Laboratories, Inc., Webster, TX), total T_3 and T_4 (Alpha Diagnostics International Inc., San Antonio, TX), and leptin (MD Bioscience Inc., St. Paul, MN; mouse leptin ELISA, IBL, Hamburg, Germany) were quantified by ELISA according to the instructions of the supplier.

Measurement of blood glucose levels

A drop of plasma sample was picked up on a test strip, and glucose level was immediately measured using the ACCU Chek compact system (Roche Diagnostics, Mannheim, Germany). Glucose concentrations were analyzed in anesthetized mice immediately after a single or the ninth stress session, and 30 min to 2 h after the termination of the last repeated stress session.

Measurement of plasma pH levels

Freshly prepared plasma samples were analyzed by pH indicator strips immediately after the last repeated stress session.

Measurement of plasma lipid composition

Total triglycerides and total cholesterol content, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) cholesterol content were analyzed by lipid-electrophoresis in the Department of Clinical Chemistry at the University Hospital of Greifswald.

Measurement of plasma amino acid composition

EDTA-plasma samples were harvested, and specimens were generated by pipetting 30-μl fresh plasma onto filter papers (No. 903; Whatman plc, Kent, UK), and were left to dry at room temperature. The amino acids were analyzed by tandem mass spectroscopy (Refurb Wallac MS2 tandem mass spectrometer; PerkinElmer, Rodgau, Germany) using a modified method of Nagy *et al.* (34) and a commercial kit (PerkinElmer, Neogram amino acids and acylcarnitines nonderivatized kit, Nurnberg, Germany).

Histological staining

To analyze lipid vacuole formation, 8-μm thick cryosections of the liver were stained by hematoxylin-eosin (HE) staining.

Lipid in cryosections of the adrenal glands (8-μm thick) was stained by the lipid-soluble dye Sudan III. Noncholesteryl esters are stained orange. Tissues were counterstained with hematoxylin.

The content of carbohydrates in the liver was analyzed in cryosections (8 μm) using a periodic acid-Schiff (PAS) staining protocol. Aldehyde groups of carbohydrates cause a purple staining.

Statistical analysis

DNA array data analysis was described earlier in *Materials and Methods*. All other statistical analysis was performed with GraphPad Prism Version 3.02 for Windows (GraphPad Software Inc., San Diego, CA). Differences between samples of stressed and nonstressed mice were analyzed by the Mann-Whitney *U* test not assuming gaussian distribution. All data in this study were expressed as mean ± SD; $P < 0.05$ was considered statistically significant.

Results

Repeated stress-induced cachexia accompanied by hypercortisolism, hyperleptinemia, and hypothyroidism

Repeated psychological stress caused a severe loss of body mass in BALB/c mice while mean food intake and water

consumption were unaltered during 4.5-d stress exposure. Food intake was 95.1 ± 19.9 g/cage in the repeatedly stressed *vs.* 91.9 ± 17.4 g/cage in the nonstressed groups, and water consumption was 250 ± 40 ml/cage in stressed *vs.* 240 ± 40 ml/cage in the control mice (three independent experiments with nine mice per cage). Given that we consistently found normal food and water intake, we questioned whether the severe loss of body mass after repeated stress exposure depends on hormonal changes. First, we found that repeatedly stressed mice showed increased corticosterone concentrations in the peripheral blood (Fig. 1A) along with a hypertrophy of the adrenal cortex with decreased size of lipid storage vesicles in the GC-producing zona fasciculata (Fig. 1, B and C). In fact, the increase in circulating GCs was less pronounced than after acute stress (results can be found in supplemental material 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), but the continuing HPA axis response might contribute to the reduction of BW of up to 20% during the time course of repeated stress exposure (Fig. 2A). This loss of body mass was not associated with significantly altered GH concentrations in the blood of stressed (13.6 ± 7.6 ng/ml) *vs.* nonstressed mice (10.8 ± 5.4 ng/ml). However, stress-induced hyperleptinemia was measured (Fig. 2B). Total T_3 (Fig. 2C) and total T_4 levels (Fig. 2D) were reduced in the plasma of stressed animals, whereas thyroglobulin concentrations remained unchanged (data not shown).

Repeated stress-induced changes of global hepatic gene expression

In an approach to a more comprehensive characterization of the metabolic changes that occur as a result of repeated acoustic and restraint stress, we recorded the expression signatures of liver from repeatedly stressed BALB/c mice and compared them with those of nonstressed controls. The Affymetrix-based mRNA expression profiling of the liver of repeatedly stressed *vs.* nonstressed animals revealed induction and repression, respectively, of 120 and 50 genes in both independent stress experiments performed (for a complete listing, see supplemental material 2). To discriminate effects of repeated stress from those of acute stress, we additionally analyzed the changes in the hepatic gene expression that occurred as a result of a single stress exposure. In this model of acute stress, 192 and 123 genes displayed stress-mediated induction or repression of expression (for a complete listing, see supplemental material 3).

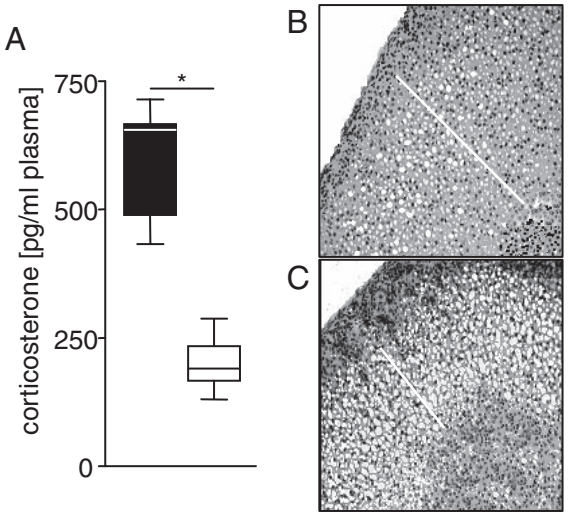


FIG. 1. Repeated stress-induced activation of the HPA axis in BALB/c mice. A, Increased plasma corticosterone levels in repeatedly stressed mice (*black box plot*) compared with nonstressed mice (*white box plot*) (n = 9 mice per group). B and C, Hypertrophy of the zona fasciculata of the adrenal cortex (*white line*) in repeatedly stressed mice (B) compared with nonstressed controls (C) (HE staining magnification, ×100); each *picture* is representative for nine mice per group. *, $P < 0.05$ Mann-Whitney *U* test; data reproduced in at least three independent experiments.

Comparatively analyzing the effects of acute and repeated stress, it became clear that both types of stress target a common set of 94 genes. Furthermore, 221 and 76 genes were predominantly regulated by acute and repeated stress, respectively (Fig. 3A).

To analyze the changes in gene expression within the framework of already accumulated knowledge, the lists of genes differentially expressed after acute and repeated stress or both were subjected to an analysis using the IPA software. This software allowed for an intuitive mining of the data of the 391 differentially expressed genes to gather an impression of the biological rationale of the expression changes experimentally observed within the context of published data.

When the IPA software was used to analyze the molecular and cellular functions targeted by stress, an influence on broad categories such as "cell growth and proliferation" and "cell death" was noted (results are shown in supplemental material 4). However, it was also apparent that genes related to metabolic diseases were most significantly influenced by the repeated stress exposure (Fig. 3B). This finding was in line with the metabolic disturbances observed before. Supporting this notion of a major impact of repeated stress on metabolism, highly significant changes were also noted for more specific categories such as amino acid metabolism and lipid metabolism. Some of these influences on metabolism were already noted during acute stress because changes related to metabolic disease ranked at number six when genes commonly influenced by acute and repeated stress were analyzed. Genes involved in more specific categories of metabolism such as lipid and amino acid metabolism were only moderately influenced by acute stress. Thus, the gene expression profiling favors the idea that acute stress sets into motion a gene regulation cascade that is then manifested during repeated stress exposure finally leading to the observed metabolic disturbances.

To elucidate the reasons for stress-induced cachexia, we decided to concentrate selectively on changes of expression of genes whose products are involved in metabolic processes and regulation of metabolic pathways.

Several genes that were regulated in the liver of repeatedly stressed animals could be linked to hypercatabolism (Fig. 3 and supplemental material 4). Genes relevant for amino acid metabolism, especially amino acid transporters and enzymes metabolizing glucogenic amino acids [Slc15a4, Slc25a15, Slc3a1, Asl, and glutamate oxaloacetate transaminase 1 (Got1)], were mostly induced (supplemen-

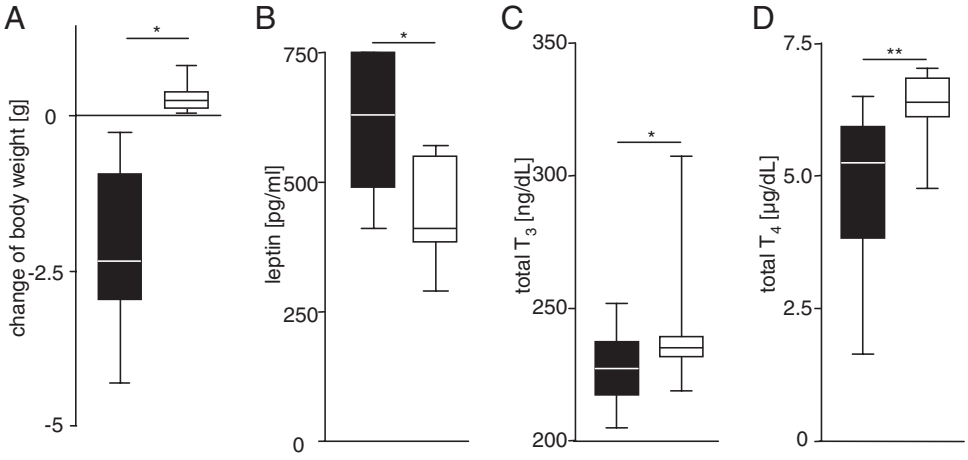


FIG. 2. Repeated psychological stress-induced loss of BW, increase of plasma leptin levels, and hypothyroidism in mice. A, Loss of body mass during the period of 4.5-d intermittent stress (*black box plots*) compared with non-stressed control mice (*white box plots*) (n = 12 mice per group). B, Plasma leptin levels after nine stress cycles compared with nonstressed mice (n = 12 mice per group). T_3 (C) and T_4 (D) concentrations in the plasma of repeatedly stressed and control mice (n = 12 mice per group). *, $P < 0.05$; **, $P < 0.01$ Mann-Whitney *U* test; data representative for two independent experiments.

tal material 2 and 4). Moreover, the gene expression profiling of the liver of repeatedly stressed mice indicated increased metabolism of lipids [Adh4, Apoa4, Cd74, Chpt1, Cyp17a1, Cyp2b10, Cyp3a13, Cyp4a10, Cyp8b1, Hsd17b2, Hsd3b2, 4632417N05Rik (Hspc105), Saa2, Slco1a1, and Xbp1].

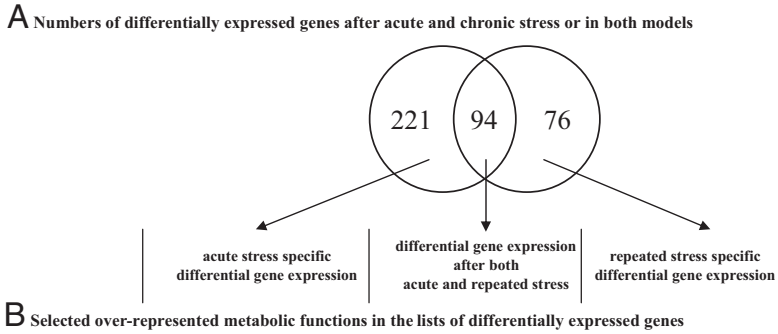
To validate the array data, we performed real-time RT-PCR focusing on chronic stress-induced dysregulation and its pathophysiological effects. Therefore, we chose genes that were associated with repeated stress-influenced metabolic processes of carbohydrate metabolism (Pck1), fat metabolism (Srebf1), and amino acid metabolism (Asl, Sds), and with stress-induced apoptosis (Gadd45b). For all selected genes, the regulation found with the Affymetrix-based expression profiling was confirmed (Table 1).

Induction of gluconeogenesis in repeatedly stressed mice

Stimulated by the observed loss in total body mass and the suspected involvement of carbohydrate metabolism, we specifically investigated the expression profiles for relevant genes, even if this category was not part of the first most significant biological functions according to the IPA categorization. Stress-induced increased expression of Foxo1, Igfbp1, Irs1, and Pck1, as well as reduced mRNA levels of Srebf1, can induce hyperglycemia because of activation of gluconeogenic pathways. In contrast, the gene products of Cebpb, Igfbp1, St3gal5, and Tnfrsf1b are associated with hypoglycemia, and may indicate counter-regulatory processes to decrease blood glucose levels.

In singularly stressed mice, *in vivo* analysis did not reveal significant changes of carbohydrate regulation pathways, *e.g.* of leptin concentrations in the plasma, blood glucose levels, or liver histology (supplemental material 1).

In contrast, repeated stress induced pathophysiologically relevant alterations of protein and lipid metabolism to provide fuel for gluconeogenesis. Only in chronically stressed mice disturbances of the carbohydrate metabolism became detectable also *in vivo*. This included a transient hypoglycemic period immediately after the termination of the ninth stress session. However, after resuming food intake in the home cage, blood glucose levels increased rapidly and resulted in a prolonged hyperglycemia that still was detectable 2 h later (Fig. 4A). In the liver of repeatedly stressed mice, an increased usage of carbohydrate reservoirs was assessed by reduced PAS staining that stains aldehyde groups of carbohydrates in tissue and revealed reduced storage of carbohydrates in the liver of repeatedly stressed mice compared with healthy control mice (Fig. 4, B and C). Moreover, after re-



B Selected over-represented metabolic functions in the lists of differentially expressed genes

Function	Rank	Rank	Rank
Metabolic Disease	26	6	1
Amino Acid Metabolism	24	62	6
Lipid Metabolism	16	16	5

FIG. 3. Impact of acute and repeated stress on metabolic genes and genes associated with metabolic disease. A, Graphical display of genes differentially expressed after acute or repeated stress, or both stress types. The *numbers* given in the Venn diagram include cDNAs, which are not functionally annotated: 29 of 221 genes regulated specifically in acute stress, nine of 94 genes regulated in both acute and repeated stress, and two of 76 genes regulated specifically for repeated stress. B, Impact of acute and repeated stress on metabolic functions. The lists of genes differentially expressed either only after acute stress and repeated stress or after both acute and repeated stress were loaded into IPA version 5.5 (Ingenuity Systems) to interpret the affected genes within the context of the published literature. Metabolism seemed to be a major target of gene regulation after repeated stress exposure, and, thus, the influence of acute and repeated stress on metabolism-associated genes was analyzed. The impact of acute or repeated stress alone as well as both stress types is displayed by showing the rankings within the list of statistically significantly overrepresented functional groups for genes related to metabolic disease, amino acid metabolism and lipid metabolism. For a complete listing of top functions that are regulated, see supplemental material 4.

TABLE 1. Real-time PCR validation of array data in repeated stress experiments

Target gene	Control group[a]	Repeated stress group[a]	*P* value[b]
Asl	2.90 ± 0.33	1.50 ± 0.23	<0.0001
Gadd45b	9.66 ± 0.64	6.83 ± 1.55	<0.0001
Pck1	2.53 ± 0.52	0.02 ± 0.72	<0.0001
Sds	5.66 ± 0.41	3.70 ± 0.64	<0.0001
Srebf1	8.01 ± 0.18	8.36 ± 0.13	<0.0001

Data represents ΔCt (target-reference Actb).

[a] Validation of expression data by real-time PCR was carried out for all individual RNA preparations of the two biological experiments (n = 9 plus n = 8 mice/group) of the two experiments focusing on effects of repeated stress exposures.

[b] Differences of ΔCt values were analyzed by Mann-Whitney test.

peated stress, insulin concentrations in the plasma were slightly increased (272.5 ± 131.4 pg/ml) compared with control mice (170 ± 149 pg/ml). Resistin, an insulin-resistance inducing adipokine, was significantly increased in the plasma of repeatedly stressed mice when compared with nonstressed animals (Fig. 4D). Last, but not least, analysis of pH in EDTA plasma samples revealed stress-induced acidosis (Fig. 4E).

Hypercholesteremia after repeated stress exposure

Global gene expression analysis of the liver of repeatedly stressed mice revealed stress-induced changes of the gene expression profile of lipid metabolism (Fig. 3 and supplemental material 2 and 4). Therefore, we started to analyze the lipid turnover of these mice. After repeated stress exposure, a hepatic steatosis was observed (Fig. 5A), whereas no significant numbers of lipid vesicles were detected in the liver of control mice (Fig. 4B). A Sudan III staining, which selectively stains triglycerides but not cholesterol esters, did not indicate any differences between stressed *vs.* nonstressed mice (data not shown). Therefore, the lipids that were accumulated in the liver were presumably not triglycerides but steroids or their precursor molecules. This is supported by the array data that showed up-regulation of genes for steroid metabolism (Cyp17a1, Cyp2b10, Cyp39a1, Cyp4a14, and Por; supplemental material 2).

Analysis of plasma lipid composition in repeatedly stressed mice showed reduced triglyceride levels (Fig. 5C) but increased total cholesterol concentrations (Fig. 5D). Among lipoproteins the HDL fraction was increased (Fig. 5E), whereas VLDL concentrations were strongly decreased (Fig. 5F). LDL-cholesterol levels did not change (Fig. 5G).

In contrast to the repeated stress model, we did not reveal differences in plasma lipid composition or histological alterations in the liver when comparing acutely stressed and control mice (supplemental material 1). In addition, the expression profiling of acutely stressed mice did not reveal major changes in genes involved in lipid metabolism, probably indicating that hepatocytes of stressed mice, started an anticipatory gene expression program during the repeated stress sessions (supplemental material 2 and 3) to face the stressful situation whose physiological impact did not become detectable until stress exposure was repeated.

Loss of essential amino acids in repeatedly stressed mice

The gene expression analysis of the liver of repeatedly stressed animals also showed altered expression profiles of genes whose products are involved in amino acid metabolism (*e.g.* Asl, Got1, Prodh, Slc15a4, Slc25a15, Slc3a1, and Tdo; supplemental material 2 and 4). Despite the small group size of analyzed animals, the amino acid composition of fresh plasma samples revealed significantly reduced concentrations of several essential amino acids, *e.g.* arginine, threonine, methionine, and tryptophan, whereas nonessential amino acids showed fewer alterations in repeatedly stressed mice (results are shown in supplemental material 5). In addition, gene expression profiling of the liver of repeatedly stressed mice showed an induction of genes for amino acid transporters and amino acid metabolizing enzymes (Sds, Slc15a4, Slc25a15, Slc3a1, Got1, and Tat), and provided hints for increased activation of amino acid degradation pathways (Aass, Ahcy, Asl, Prodh, and Tdo2). The induction of Asl expression (supplemental material 2) along with a loss of arginine and citrulline in the plasma (supplemental material 5) provided hints for altered urea cycle activity. This substantiates the observations of systemic usage of the body's protein stores in repeatedly stressed BALB/c mice. In contrast, after a single acute stress session, we found induction of mRNA expression of only a few glucogenic amino acid transporters and metabolizing enzymes in the liver (Sds, Slc38a2, and Tat), which did not result in altered amino acid levels in the periphery (data not shown).

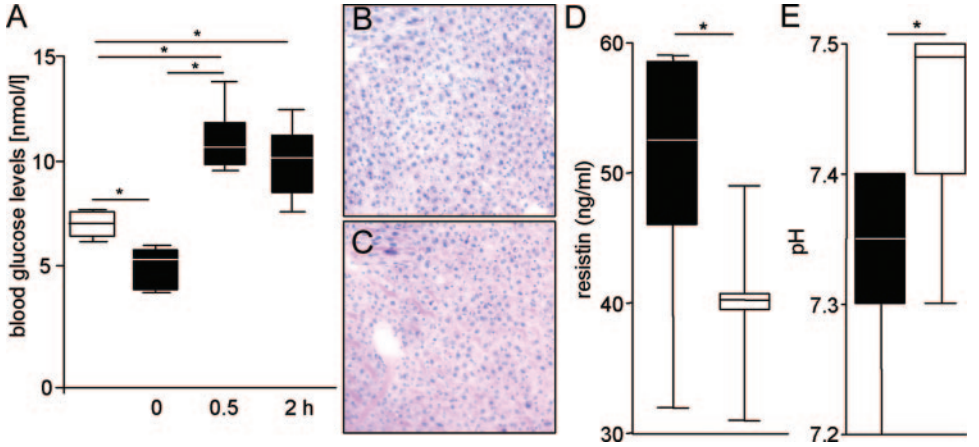


FIG. 4. Disturbances of murine carbohydrate metabolism after repeated psychological stress. A, Kinetics of blood glucose levels immediately after termination of the ninth stress cycle (*black box plots*) compared with controls (*white box plot*) (n = 9 mice per group). B and C, Reduced storage of carbohydrates in the liver of repeatedly stressed mice (B) compared with nonstressed mice (C) (PAS staining magnification, ×200); each *picture* is representative for nine mice per group. Plasma resistin levels (D) and pH of EDTA plasma (E) of stressed and nonstressed mice (n = 12 mice per group). *$P < 0.05$ Mann-Whitney *U* test; data representative for two independent experiments.

Additional expression data

Finally, we found a number of other stress-induced alterations of hepatic gene expression. Several genes that were regulated in the liver of repeatedly stressed mice provided hints for cell death (*e.g.* Gadd45b, Cdkn1a, and Ccnd1; Table 1 and supplemental material 2 and 4, A and B). These alterations are currently studied in our laboratory in more detail.

Among the genes that were selectively regulated in repeatedly stressed mice but not after a single stress exposure, we found evidence for increased local steroidogenesis in the liver (*e.g.* Cyp17a1, Hsd17b2, and Hsd3b2) and heightened bile acid production (Cyp2b10, Cyp8b1). Plasma taurine levels in these animals showed a slight tendency to be reduced (553.57 ± 91.24 nM in repeatedly stressed *vs.* nonstressed mice 631.66 ± 33.13 nM). The gene of the taurine transporter Slc6a6 in turn was repressed in liver tissue after a single stress session and remained down-regulated also in repeatedly stressed animals (supplemental material 2 and 3).

In addition, we found stress-induced changes in mRNA expression of genes related to detoxification of radicals, acute phase response, and immune regulation. The biological relevance of these data is currently analyzed in further *in vivo* experiments.

Discussion

BALB/c mice that are highly susceptible to psychological stress recently were shown to suffer from an impaired antibacterial defense, and from depression-like behavior within a period of 4.5-d combined acoustic and restraint stress (8, 14).

Here, we show that these mice also developed a hypermetabolic syndrome resulting in a severe loss of total body mass.

Our data reveal that already a single acute stress exposure caused profound changes in hepatic gene expression. Genes important for metabolic pathways regulating the carbohydrate turnover showed stress-induced alterations of mRNA expression in hepatic tissue. An acute stress response is essential for energy mobilization to "fight or flight" in a potentially harmful situation and to sustain or reconstitute allostasis (1). Initially, catecholamines activate glycogenolysis, gluconeogenesis, and accelerate lipolysis that subsequently is assisted by catabolic GC-induced pathways (4, 5, 12, 32, 35). Acute psychological stress in our model was associated with activation of the stress axes, and an induction of the expression of gluconeogenic genes and of transporters for glucogenic amino acids (Pck1, G6pc, Slc37a4, Slc15a4, Slc25a15, Slc38a2, Slc3a1, Sds, Slc6a6, and Tat).

When stress remained a singular event, we did not find significant changes in metabolic parameters (results are shown in supplemental Fig. 1), but when stress was repeated, female BALB/c mice developed severe systemic neuroendocrine and metabolic alterations. Several researchers found that repeated acute restraint stress causes a loss of BW in rodents (36–38), mainly mediated by initially increased energy expenditure and reduced food intake that normalized or even heightened within a few days after starting repeated stress due to neuroendocrine adaptations (36, 38). Others showed prolonged lowered food intake during 4.5-d repeated restraint stress due to prolonged neuroendocrine dysregulation (37). In our study we found no changes of total food consumption during 4.5 d in repeatedly stressed animals compared with nonstressed controls, which may result from an initially reduced food intake after the first stress session and increased food intake in the later phase of repeated stress exposure in which we additionally found manifested neuroendocrine and metabolic dysregulation. Repeatedly stressed mice suffered from an increase in metabolic rate in excess of the normal metabolic response. Such a hypermetabolic response leads to a marked increase in energy demands. Protein inappropriately becomes an energy source, and increased use of protein rapidly depletes lean body mass. A hypercatabolic response is a typical feature in infection, cancer, and prolonged critical illness, and goes

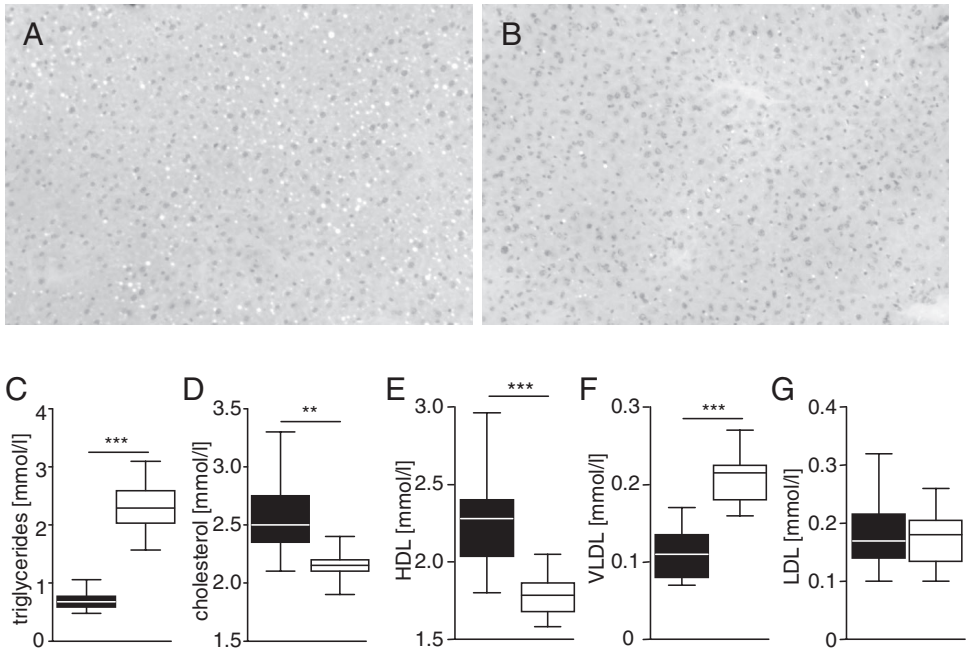


FIG. 5. Disturbances of fat metabolism in repeatedly stressed mice. A and B, Hepatic steatosis in repeatedly stressed mice (A) compared with nonstressed mice (B) (HE staining magnification, ×20); each *picture* is representative for nine mice per group. C–G, Plasma fat composition of repeatedly stressed mice (*black box plots*) and nonstressed controls (*white box plots*); triglyceride levels (C), total cholesterol (D), HDL-cholesterol (E), VLDL-cholesterol (F), and LDL-cholesterol (G) were measured immediately after the ninth stress session (n = 12 mice per group).**, $P < 0.01$; ***, $P < 0.001$, Mann-Whitney *U* test.

along with fever, dysregulations of the cardiovascular system, hyperglycemia, dyslipidemia, accelerated proteolysis, tissue damage/cell death, perfusion disturbances, and invasion by microorganisms (2, 3, 6, 15–18, 30, 39). In contrast, starvation is connected with diminished food intake, hyperthyroidism, and reduced protein catabolism (3, 16, 40). During a hypercatabolic response as in our repeated stress model, food intake is often normal (3, 16, 40) and associated with hypothyroidism (2). Recently, it was shown that prolonged sleep deprivation also can cause such a hypermetabolic response in rats (19, 20). The stress experiments in our model were performed in the recovery phase of the animals, and sleep deprivation may have affected metabolic functions. Both repeatedly stressed mice and long-term sleep deprived rats showed lowered total T_3 and T_4 levels that did not depend on altered TSH concentrations (20). Koban and Swinson (20) propose a reciprocal relationship of catecholamines, which progressively increase during sleep deprivation and thyroid hormone concentrations that decline continuously in prolonged reduction of sleep time. However, in contrast to our repeatedly stressed mice that showed unaltered food intake, sleep-deprived rats were hyperphagic while body mass was massively consumed (19, 20). They did not find altered GC levels, whereas chronic psychological stress characteristically was associated with persistently high corticosterone concentrations in the plasma.

The activation of the central nervous system can profoundly affect metabolic regulation such as shown for the thyroid hormone release (20). The target organ of metabolic regulatory pathways is predominantly the liver (1, 4, 41). The activation of the HPA axis with increased GC levels can stimulate food intake and activate carbohydrate, fat and protein catabolism. In turn, the brain receives signals such as actual glucose and lipid concentrations or increased energy demand (1, 5, 42). In consequence, central nervous system activation induces regulatory pathways that equilibrate metabolism to supply the needed energy, *e.g.* increasing glucose formation in the liver (1, 4, 42).

Hypercortisolism shifted metabolic functions toward carbohydrate, lipid, and protein catabolism (23–25, 37–43) to sustain energy supply by replenishing glucose that is the main energy source of the body. Glucose can be released after glycogenolysis or induction of gluconeogenesis when the supply with food is insufficient. Primarily, alanine and glutamate are precursor molecules for gluconeogenesis (23–25, 43–46). Hepatic induction of alanine aminotransferase (Gpt) 2 and Got1 may supply the metabolites for glucose synthesis (23–25, 44). Thereafter, alanine and glutamate can be reconstituted by biotransformation, whereas essential amino acids cannot be replenished by biosynthesis in repeatedly stressed mice (45, 46). Deamination of amino acids results in the production of ammonia, which is detoxified in the liver by the urea cycle (43, 46, 47). Increased expression of the urea cycle enzyme Asl in the liver of repeatedly stressed mice along with usage of arginine and citrulline as intermediate products of the urea cycle indicates heightened stress-induced ammonia detoxification to provide C bodies of amino acids for metabolic pathways such as gluconeogenesis. Lactate, which alternatively can serve as substrate for hepatic gluconeogenesis, was produced in high amounts in peripheral tissues during hypermetabolism such as during sepsis and can cause lactic acidosis (6, 23, 25, 28, 48, 49). Acidosis that was detectable in repeatedly stressed mice is often paralleled with insulin resistance and hyperglycemia (2, 17, 48). In fact, in repeatedly stressed mice, we found increased concentrations of the adipokine resistin, which is inducible by GCs, prolactin, and GH, and has been identified to lower insulin sensitivity (45). Prolonged hyperglycemia, especially in critically ill patients, increases the risk of infectious complications, neuronal damage, and multiorgan dysfunction syndrome (17, 18, 36, 39, 49). In line with this, repeatedly stressed mice suffered from a reduced antimicrobial response (8, 14). Lam *et al.* (42) showed that increased glucose sensing by the brain and elevated intracerebral concentrations of lactate reduced the hepatic secretion of VLDL-cholesterol, and caused a decline of plasma triglyceride levels in rodents. In addition, Ricart-Jané *et al.* (37) found that repeated restraint stress caused a loss of plasmatic triacylglycerols with VLDL levels, which was accompanied by a reduced food intake. Heightened triglyceride clearance and increased lipolysis to assemble C3 bodies for gluconeogenesis can result in essential fatty acid deficiency (50–52).

In our stress model, such hypotriglyceridemia went along with hypercholesteremia and up-regulation of GC-sensitive cytochrome genes in the liver (51–53). Cyp4a enzymes are involved in the removal of fatty acids and can counter-regulate hepatic steatosis, which we found to be a typical consequence of repeated stress exposure in mice. Increased expression of Cyp17a1 gives hints for hepatic induction of steroidogenesis (52) in repeatedly stressed mice, and up-regulation of expression of Cyp39a1 and Cyp2b10 indicates increased removal of steroids by bile acid production (53). Importantly, the cholesterol metabolism of rodents and humans is difficult to compare. In mice HDL is the main lipoprotein present in the blood and essentially required for steroidogenesis (37, 54), whereas humans use LDL for steroid synthesis and have lower HDL concentrations (54, 55). Furthermore, HDL is able to scavenge endotoxins from the plasma (56). The relevance of stress-induced elevation of plasma HDL levels in mice, *e.g.* for steroid synthesis or scavenging the bacterial compound lipopolysaccharide, remains to be elucidated. Other authors found that lipopolysaccharide or TNF challenges particularly increase the catabolic rate (57, 58). Such effects seem to be mediated via TNF-induced neuroendocrine stimulation, *e.g.* activation of the sympathetic nervous system that primarily causes glucose formation (4, 5, 12, 32, 35, 58–61). In addition, release of catecholamines potentially induces the expression of the leptin gene in adipose and hepatic tissue (61–63). Leptin then afferently can signal to the brain to sustain inhibitory effects on food intake. In this relation, one should expect reduced food consumption in the hyperleptinemic repeatedly stressed mice. However, we did not find altered food intake, whereas total body mass furthermore was lost. One possible explanation for the drastic loss of BW along with hyperleptinemia is that leptin potentially can increase the energy expenditure such as enhancing the activation of the respiratory chain and, therefore, can increase energy consumption (64, 65). In this study we showed that already acute stress can induce drastic changes of hepatic gene expression that did not significantly disrupt allostatic regulation of metabolism in mice. In turn, repeated psychological stress in BALB/c mice along with systemic immunodeficiency that was reported previously (8, 66) induced a hypermetabolic stress syndrome.

It is not clear why some individuals during prolonged stressful situations lose weight, whereas others gain body mass (2, 3, 7, 10, 15–24). Because there is an increased number of patients suffering from metabolic syndrome and clinically relevant associated illness, many publications show that chronic stress is promoting the development of a metabolic syndrome that is associated with gain of fat mass (obesity), type 2 diabetes, hyperlipidemia, and hypertension (3, 5, 7). In contrast, in our animal experiments with BALB/c mice as a mouse strain with high-stress susceptibility (8), we found metabolically driven wasting because of a hypercatabolic stress response. We assume that the genetic predisposition influences the development of either stress-induced metabolic syndrome or loss of BW phenotype. Moreover, it is shown that besides genetic predisposition, environmental factors influence prenatal and postnatal neuronal and neuroendocrine differentiation, resulting in different coping styles in the adult (67). They showed that proactive/aggressive animals develop stress-induced hypertension, cardiac arrhythmias, and inflammation, whereas reactive/passive individuals are more susceptible to anxiety disorders, metabolic syndrome, depression, and infection. However, the neurobiology and endocrine regulation of these different coping styles are not well understood, yet. A loss of biological reserves as in our model of repeated stress exposure is as fatal as the development of a metabolic syndrome because of losing the ability to fight infection and cancer (3, 16, 21–24).

In the clinical setting, it is now clear that catabolic response will become autodestructive if not contained. The severity of complications will occur in proportion to lost body protein. In our model, particularly arginine deficiency became evident. Interestingly, alimentation with arginine and omega-3-fatty acids-enriched enteral feeds decreased hospital days and infectious complications in critically ill patients (68), which commonly show a loss of about 10% of lean body mass (63–65). Healthy adults require about 0.8 g protein/kg BW·d to maintain homeostasis. Stressful events such as traumatic injury or infection increase the body's protein requirement up to 1.5–2 g protein/kg BW·d or even more. However, humans cannot metabolize more than 2 g/kg BW·d. This often results in a fatal negative nitrogen balance in severely ill patients (69). Finally, amplified protein breakdown with a loss of more than 40% of lean body mass leads to irreversible cell damage (68–71).

Here, we show that highly demanding psychological stress in the absence of injury or infection is able to induce a severe hypermetabolic syndrome in mice. Such overwhelming wasting condition can reduce the individual's resistance to further stressful stimuli, such as injury or infection.

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