Acute Psychological Stress Accelerates Reverse Cholesterol Transport via Corticosterone-Dependent Inhibition of Intestinal Cholesterol Absorption

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Rationale: Psychological stress is associated with an increased risk of cardiovascular diseases. However, the connecting mechanisms of the stress-inducing activation of the hypothalamic-pituitary-adrenal axis with atherosclerosis are not well-understood.

Objective: To study the effect of acute psychological stress on reverse cholesterol transport (RCT), which transfers peripheral cholesterol to the liver for its ultimate fecal excretion.

Methods and Results: C57Bl/6J mice were exposed to restraint stress for 3 hours to induce acute psychological stress. RCT in vivo was quantified by measuring the transfer of [1H]cholesterol from intraperitoneally injected mouse macrophages to the lumen of the small intestine within the stress period. Surprisingly, stress markedly increased the contents of macrophage-derived [1H]cholesterol in the intestinal lumen. In the stressed mice, intestinal absorption of [14C]cholesterol was significantly impaired, the intestinal mRNA expression level of peroxisome proliferator-activated receptor-α increased, and that of the sterol influx transporter Niemann-Pick C1-like 1 decreased. The stress-dependent effects on RCT rate and peroxisome proliferator-activated receptor-α gene expression were fully mimicked by administration of the stress hormone corticosterone (CORT) to nonstressed mice, and they were blocked by the inhibition of CORT synthesis in stressed mice. Moreover, the intestinal expression of Niemann-Pick C1-like 1 protein decreased when circulating levels of CORT increased. Of note, when either peroxisome proliferator-activated receptor α or liver X receptor α knockout mice were exposed to stress, the RCT rate remained unchanged, although plasma CORT increased. This indicates that activities of both transcription factors were required for the RCT-accelerating effect of stress.

Conclusions: Acute psychological stress accelerated RCT by compromising intestinal cholesterol absorption. The present results uncover a novel functional connection between the hypothalamic-pituitary-adrenal axis and RCT that can be triggered by a stress-induced increase in circulating CORT. (Circ Res. 2012;111:1459-1469.)

Key Words: atherosclerosis ■ corticosterone ■ liver X receptor α ■ peroxisome proliferator–activated receptor α ■ reverse cholesterol transport ■ stress

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is the endocrine hallmark of stress. The end hormones of the HPA axis, the glucocorticoids, are the main humoral mediators of stress, and their increased secretion results in an intricate cascade of homeostatic mechanisms that facilitate survival. Glucocorticoids released during stress enhance resistance to sepsis in mice, and they are important modulators of allergic inflammatory responses in mice and humans. In rodents, various experimental models have been applied to mimic the conditions of acute or chronic psychological stress experienced by humans. Among them,
physical restraint stress is the most widely applied procedure. This and other types of stress-inducing procedures have been applied when searching for mechanisms of psychological stress linked to atherosclerosis. Interestingly, when genetically atherosclerosis-susceptible apolipoprotein (apo) E-deficient mice were exposed to various types of stress-inducing procedures, the development of atherosclerosis either accelerated or remained unaffected.

A key event of atherosclerosis is the generation of cholesterol-loaded macrophage foam cells in the arterial intima. High-density lipoprotein (HDL) particles, by stimulating efflux of excessive cholesterol from macrophages and promoting its transfer to the liver for its ultimate excretion in feces, initiate the macrophage-specific reverse cholesterol transport (M-RCT), which is considered a critical antiatherogenic pathway. Importantly, the rate of the M-RCT pathway in vivo can be modulated at any of its 3 key steps, namely, macrophage cholesterol efflux to HDL, hepatic uptake of HDL cholesterol, and ultimate excretion of macrophage-derived cholesterol by the intestine.

In the present study, we examined whether acute psychological stress modifies the rate of M-RCT. For this purpose, we exposed mice to physical restraint stress for 3 hours and evaluated M-RCT within the stress period using a recently validated assay. Surprisingly, we found that a single period of acute stress accelerated the rate of M-RCT. The mechanism was found to be mediated by elevation of circulating corticosterone (CORT). Moreover, peroxisome proliferator–activated receptor-α (PPARα) was identified as a transcription factor that downregulated the intestinal expression of the cholesterol transporter Niemann-Pick C1–like 1 (NPC1L1), and so decreased cholesterol absorption by the enterocyte.

### Results

#### Serum Lipids, HDL, and HPA Axis Components in Stressed Mice

Serum parameters of stressed mice placed in restrainers for 3 hours and of the matched nonstressed control group are summarized in Online Table I. Levels of the stress hormone CORT increased significantly (5-fold) in serum of the stressed mice. Total and HDL cholesterol were reduced, but no significant differences were found in apoA-I, triglycerides, lecithin-cholesterol acyltransferase activity, or in the potential of serum to generate preβ-HDL between stressed and control mice. Fractionation of serum by fast pressure liquid chromatography showed that exposure to stress did not change the elution profile of HDL (Online Figure I). The time course of stress-induced effects in HDL particle size and in serum levels of total cholesterol, apoA-I, corticotropin-releasing factor (CRF), the principal regulator of the HPA axis, and CORT were further studied in a separate experiment (Online Figure II). HDL particle size remained unchanged (approximately 11 nm) over the entire 3-hour period of stress (Online Figure IIA). Serum cholesterol levels tended to decrease within 10 minutes of exposure to stress without reaching a statistically significant difference at any time point when compared with the basal level, whereas apoA-I levels remained essentially identical throughout the entire stress period (Online Figure IIB). In contrast, stress induced distinct changes in the circulating levels of HPA axis hormones. Although both CRF and CORT rapidly increased on exposure to stress, CRF peaked at 10 minutes and then rapidly returned to basal levels, whereas CORT levels remained elevated until the 3-hour time point (Online Figures IIC and IID). This dual effect is characteristic of stress and is an essential part of the feedback regulation of the HPA axis.

### Methods

For a detailed description, see the Online Data Supplement.

C57Bl/6j mice were either exposed to restraint stress by being placed into well-ventilated plastic restraint cylinders for 3 hours or deprived of food and water in their cages for the 3-hour period (control mice). In some experiments, mice were exposed to 3 sessions of 3-hour stress within 24 hours or to 1 to 2 sessions per day of 2-hour stress for periods of 5 to 7 days. In all protocols, the stress was applied during the light cycle. M-RCT in vivo was evaluated in stressed mice and their nonstressed controls within the 3-hour period. For the general in vivo M-RCT assay, mice received intraperitoneal injections of [3H]cholesterol-loaded J774 macrophages and, after 3 hours, radioactivity in serum, liver, and intestinal contents were measured, as described previously. The effect of stress on the M-RCT also was evaluated in peroxisome proliferator-activated receptor (PPARα) knockout (KO), liver X receptor (LXR) α/KO, human apoA-II transgenic, and in wild-type C57Bl/6j mice fed with ezetimibe-supplemented or GW0742-supplemented diets, or under pharmacological treatments that affect glucocorticoid production by the adrenal glands in vivo. The ability of serum to accept foam cell–derived cholesterol was evaluated ex vivo, the hepatic uptake of intravenous administered [3H]cholesterol oleate-labeled HDL was assessed over 3- and 24-hour periods, and intestinal cholesterol absorption was evaluated in vivo with a fecal dual isotope method adjusted for the 3-hour timeframe. Synthesis of cholesterol in the liver and in the intestine was evaluated by intraperitoneal administration of tritiated water. Expression of selected transcription factors and cholesterol transporters genes was analyzed by quantitative real-time polymerase chain reaction (RT-PCR) in J774 macrophage foam cells, and in the liver and the small intestine of control and stressed mice. Expression of intestinal Niemann-Pick C1-like 1 (NPC1L1) and hepatic scavenger receptor-BI (SR-BI) proteins was analyzed by Western blotting. All values are expressed as mean ± standard error of the mean.

### Rate of M-RCT in Stressed Mice

To investigate whether a short period of stress would affect the rate of M-RCT, mice were placed in restrainers immediately after having received an intraperitoneal injection of [3H] cholesterol-loaded macrophages, and the transfer of the tracer to HDL, liver, and the intestinal contents was evaluated after a

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**Non-standard Abbreviations and Acronyms**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<td>CORT</td>
<td>corticosterone</td>
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<td>CRF</td>
<td>corticotropin releasing factor</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
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<td>KO</td>
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<td>LXR</td>
<td>liver X receptor</td>
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<td>M-RCT</td>
<td>macrophage-specific reverse cholesterol transport</td>
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<td>NPC1L1</td>
<td>Niemann-Pick C1–like 1</td>
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<td>PPAR</td>
<td>peroxisome proliferator–activated receptor</td>
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<td>RT-PCR</td>
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3-hour period of stress and expressed as percentages of the injected dose (Figure 1). In the stressed group, the macrophage-derived radioactivity was lower in serum HDL (Figure 1A) and remained unchanged in the liver (Figure 1B). Of note, in the stressed mice, the 3H-radioactivity levels in the luminal contents of the small intestine were markedly elevated, particularly in the free cholesterol fraction (Figure 1C), whereas no significant differences were observed in the minute amounts of radioactivity found in the secreted bile acids, as compared with the nonstressed control mice. These results indicated that exposure to stress had accelerated the M-RCT rate in the mice. Moreover, the M-RCT-accelerating effect of stress also was observed after intraperitoneal injection of other types of 3H cholesterol-loaded macrophages, (ie, either primary mouse peritoneal macrophages or murine macrophage-like P388D1 cells) (Online Figures IIIA and IIB), which demonstrated that the type of macrophage had not influenced M-RCT. To exclude the possibility that the injection of the cells into a site adjacent to the intestine had resulted in increased cholesterol transport via peritoneal circulation with direct intestinal uptake, we evaluated the transfer of J774-derived 3H cholesterol to the intestine after a subcutaneous injection of the cells in the scapular region of the mice. Because the 3-hour period was too short to evaluate the cholesterol movement from the skin to the intestine, the mice were exposed to 3 sessions of 3-hour stress within 24 hours after subcutaneous injection of 3H cholesterol-loaded macrophages, followed by M-RCT evaluation (Online Figure IIC). Similar to the results obtained when the macrophages were intraperitoneally injected, M-RCT from subcutaneously injected cells increased during stress, so excluding the possibility that the intraperitoneal injection had induced a bypass of 3H cholesterol from the injected macrophages to the intestine. Taken together, the results demonstrated that stress accelerated the transfer of macrophage-derived cholesterol to the intestine. This unexpected finding prompted us to explore which of the key steps along the M-RCT pathway was specifically affected during the stress period.

**Macrophase Cholesterol Efflux to Mouse Serum**

We first studied the effect of stress on the functionality of the endogenous cholesterol acceptors and the efficiency of macrophages to release cholesterol (Figure 2A). Analysis of cholesterol efflux from 3H cholesterol-loaded J774 cells incubated for 3 hours in media containing serum from control or stressed mice showed that stress did not modify the cholesterol efflux capacity of serum (Figure 2A, left). Both sera were also equally efficient in promoting cholesterol efflux from 3H cholesterol-loaded C57Bl/6J mouse peritoneal macrophages isolated from control mice (Figure 2A, right). These results were consistent with the similar levels of apoA-I found in sera from stress and control mice (Online Table I). Because increased serum CORT concentration facilitates its diffusion into the peritoneal cavity (in basal conditions approximately 20% of serum CORT concentration is present in peritoneal fluid), we mimicked the CORT-enriched peritoneal fluid of stressed mice by performing an in vitro cholesterol efflux assay in the presence of CORT. However, adding CORT (50 and 250 ng/mL) to the culture media did not affect the ability of J774 macrophages to release cholesterol to serum (Online Figure IV). Overall, the results are consistent with the view that the stimulatory effect of stress on the M-RCT pathway does not occur at its initiation, but rather at its terminal steps.

**Hepatic Uptake of HDL-Derived Cholesterol**

Next, we evaluated the effect of stress on the hepatic uptake of HDL cholesterol. To this end, HDL preparations derived from both control and stressed mice were labeled in parallel. Autologous 3H cholesterol oleate-labeled HDL were intravenously administered to mice immediately before exposure to stress and to control mice. After 2 minutes of injection, the radioactivities in serum were similar in the 2 groups of mice, being within the range of 72% to 85% of the dose. In contrast, a moderate but statistically significant increase in hepatic radioactivity was found after 3 hours of injection in the stressed mice relative to the control group (Figure 2B). The finding that stress enhanced hepatic uptake of HDL-derived cholesterol...
HDL cholesterol clearance, fractional catabolic rate of \[3H\]cholesterol in serum was determined in both groups of mice. After 3 hours of sterol administration, the \[14C]/\[3H\] ratio was measured in the luminal contents recovered from the stomach, and significantly enhances RCT in mouse models. In line with a previous report, ezetimibe significantly increased the \[14C]/\[3H\] ratio of cholesterol. For this aim, \[14C\]cholesterol and the nonabsorbable \([\mathrm{H}]\) sitostanol were administered by gastric gavage immediately before the stress period, and the radioactivities in the contents of the small intestine were measured after 3 hours. The transit time of the administered sterols from the stomach to the small intestine was not affected by stress, as reflected by a similar recovery of \H\-radioactivity derived from \(\beta\)-sitostanol in the small intestine of both groups (Online Figure VII). However, as reported previously, the colonic motility increased after exposure to restraint stress, which resulted in higher levels of sitostanol in feces. To normalize cholesterol absorption relative to the transit rate along the entire intestine, we used the \([\mathrm{C}]\)/\([\mathrm{H}]\) ratio in serum of the stressed mice were markedly lower than in control mice (Figure 2C, right). These results indicate that stress reduced the intestinal absorption of cholesterol.

Because NPC1L1 is the critical transporter for cholesterol absorption in the intestine, we also examined the effect of stress on M-RCT in mice treated with ezetimibe, which efficiently blocks the NPC1L1-dependent cholesterol absorption pathway and significantly enhances RCT in mouse models. In line with a previous report, ezetimibe significantly increased the transfer of macrophage-derived \H\-radioactivity to the small intestinal lumen. However, no additive increase of the M-RCT rate occurred in ezetimibe-treated mice exposed to stress (P=0.54; Figure 3A). Similarly, in mice treated with the PPAR\(\delta\) agonist GW0742, which reduces NPC1L1 mRNA expression in mice, a 2-fold increase was found in the tracer content in small intestine, and, importantly, stress did not have any further effect on M-RCT rate (P=0.36; Figure 3B). Altogether, the data suggest that stress and the 2 drugs share a common M-RCT stimulatory mechanism that involved inhibition of the NPC1L1 function in the intestine, thus impairing the reabsorption of HDL-derived cholesterol was redirected toward the liver without a net effect on HDL clearance. A similar effect was recently found during PPAR\(\gamma\) activation, which redirected cholesterol from the liver toward adipose tissue, without having any influence on HDL cholesteryl ester turnover.

### De Novo Synthesis of Cholesterol in the Liver and the Small Intestine

We analyzed whether the enhanced intestinal excretion of cholesterol induced by stress stimulated its hepatic or intestinal biosynthesis as a compensatory response. For this purpose, mice were intraperitoneally injected with \(\text{H}_2\text{O}\) and then exposed to stress. After 3 hours, cholesterol was extracted from the liver and the small intestine and de novo synthesis of cholesterol in these tissues was calculated (Online Figure VI). The cholesterol synthesis rate in the liver of control mice was in good agreement with earlier studies, and exposure to stress led to a non-significant trend toward an increase, whereas no change in the rate of cholesterol synthesis in the small intestine was observed.

### Cholesterol Absorption Function of the Intestine

Given the importance of the intestine in the net RCT flux, we next investigated the effect of stress on the intestinal absorption of cholesterol. For this aim, \[\mathrm{C}\] cholesterol and the nonabsorbable \[\mathrm{H}\] \(\beta\)-sitostanol were administered by gastric gavage immediately before the stress period, and the radioactivities in the contents of the small intestine were measured after 3 hours. The transit time of the administered sterols from the stomach to the small intestine was not affected by stress, as reflected by a similar recovery of \H\-radioactivity derived from \(\beta\)-sitostanol in the small intestine of both groups (Online Figure VII). However, as reported previously, the colonic motility increased after exposure to restraint stress, which resulted in higher levels of sitostanol in feces. To normalize cholesterol absorption relative to the transit rate along the entire intestine, we used the \[\mathrm{C}\]/\[\mathrm{H}\] ratio in serum of the stressed mice were markedly lower than in control mice (Figure 2C, right). These results indicate that stress reduced the intestinal absorption of cholesterol.

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cholesterol. Because NPC1L1 affects cholesterol absorption, but not bile acid absorption, this suggestion is compatible with the observed increase in [3H]cholesterol, but not in [3H]bile acids, in the intestinal lumen of the stressed mice (Figure 1C).

**Expression of Genes Related to Cellular Cholesterol Transport**

To search for potential mechanisms mediating the effect of stress on M-RCT, we determined by quantitative RT-PCR the mRNA expression of PPARα, PPARδ, and LXRα, which are major regulators of cholesterol homeostasis, and their downstream cholesterol transporter target genes, which are important for RCT28,29 (Figure 4). Gene profiles were analyzed in J774 macrophages that had been incubated for 3 hours in CORT-enriched culture medium and in livers and small intestines derived from stressed mice. A 3-hour exposure to CORT induced LXRα expression in the macrophages, but no difference was found for PPARα, or for ATP-binding cassette transporters (ABC) A1 and G1, the 2 pivotal transmembrane proteins determining cholesterol efflux in macrophages30 (Figure 4A). Compared with control mice, mice exposed to stress had significantly upregulated PPARα mRNA expression levels in the liver and the small intestine, whereas no differences were found for LXRα expression in these two tissues (Figures 4B and 4C). The levels of PPARδ mRNA also remained unchanged in the small intestines of the stressed mice (Figure 4C). No statistically significant differences between control and stressed mice were found in hepatic ABCG5/G8 heterodimer, or in SR-BI and hepatic lipase (HL) mRNA levels (Figure 4B). Of note, NPC1L1 mRNA expression was significantly downregulated in the intestines of stressed mice, whereas expression of ABCG5/G8 and ABCA1, which favor cholesterol export from absorptive cells into the lumen or to the basolateral compartment,31 respectively, remained unchanged (Figure 4C). Overall, the results strongly indicated a functional connection between acute stress and decreased function of intestinal NPC1L1 via a mechanism likely to be mediated by PPARα, which is known to negatively regulate NPC1L1 expression in mice.32
Identification of CORT as a Mediator Involved in the Stimulatory Effect of Stress on M-RCT

The finding that serum concentration of CRF, the initial HPA axis coordinator and essential mediator of endocrine and behavioral stress responses, declined to the basal levels after 10 minutes of stress suggested that the stress mediator of the RCT response was most likely located downstream of CRF. One possibility is that the typical stress-dependent elevation of the circulating hormone CORT could be the signal triggering a sequence of events leading to the inhibition of intestinal cholesterol absorption. To test this hypothesis, we administered CORT to control mice and evaluated M-RCT after 3 hours (Figure 5). To keep high levels of serum CORT during the entire study period, 2 subcutaneous doses of CORT (5 mg/kg) or vehicle (control group) were administered, one immediately before and the other 1.5 hours after the intraperitoneal injection of [3H]cholesterol-loaded macrophages. Three hours after the initial CORT injection, the mice were euthanized and M-RCT was evaluated. Administration of CORT to control mice maintained high levels of CORT in serum throughout the 3-hour experimental period (Online Table II) similar to those found in mice maintained in restrainers for the same period of time. Serum levels of CRF were significantly reduced in the CORT-treated mice possibly because of the inhibitory effect of the sustained high levels of serum CORT achieved by the administration of exogenous CORT. No differences in HDL cholesterol were found between the groups (Online Table II). Of note, mere administration of CORT to control mice fully reproduced the stress effect on the transfer of macrophage-derived [3H]cholesterol to the various compartments analyzed. Importantly, the significant increase in the [3H]-radioactivity levels in the intestinal contents of the CORT-treated mice indicated that sole administration of CORT was sufficient to accelerate the rate of M-RCT in the nonstressed mice. Because CRF, but not CORT, increases colonic motility during stress,33 these results imply that factors regulating the stress effects on intestinal transit and on M-RCT were independent, and also suggested that CORT contributed to the inhibition of intestinal cholesterol absorption in the stressed mice. To verify the putative role of CORT, we further evaluated whether blocking adrenal CORT synthesis could prevent the effect of stress on M-RCT. To this end, mice received a single intraperitoneal injection of the cytochrome P450 inhibitor metyrapone (100 mg/kg) 1.5 hours before exposure to stress. The rate of M-RCT to the intestinal lumen was evaluated within the 3-hour stress period (Figure 6). Because metyrapone alone did not modify the transfer of [3H]cholesterol from macrophages to the small intestinal contents relative to vehicle-treated control mice (0.46±0.05% versus 0.51±0.11% of the injected dose; P = 0.58), we used metyrapone-treated control mice and vehicle-treated stressed mice as reference groups. The selected dose of metyrapone fully inhibited the increase in serum CORT induced by stress (Figure 6A). More importantly, the acceleration of M-RCT induced by stress was fully inhibited in mice that had been pretreated with metyrapone (Figure 6B). We also determined the effect of stress on M-RCT in human apoA-II transgenic mice, which have been shown previously to have HDL and apoA-I deficiencies and selective depletion of adrenal cholesterol20 (Online Table III and Online Figure VIII). These mice, when exposed to stress, exhibited impaired CORT production (Online Figure IXA) similar to that found in apoA-I KO mice.24 Consistent with the observed involvement of CORT in the M-RCT response to stress, no significant change in the M-RCT rate was found by exposing these mice to stress (Online Figure IXB). Altogether, the results implicated CORT as playing a significant role in the stress-triggered M-RCT response. The finding of a significant positive correlation between CORT levels and M-RCT rate (Online Figure XA) further supported this notion. Moreover, this novel stress-dependent effect on RCT and its significant correlation with circulating CORT also were observed after repeated exposure of mice to acute periods of stress for 1, 5, or 7 days (Online Figure XB). In these groups of mice, macrophage-to-feces RCT also was significantly accelerated (Online Figures XC and XD).

Mechanisms Involved in the CORT-Mediated Effect on M-RCT

Next, we investigated the role of CORT in the stress-related modulation of intestinal gene expression. For this purpose, we measured by quantitative real-time polymerase chain reaction the expression levels of the transcription factors PPARα,
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PPARα, and LXRα in CORT-treated control mice and metyrapone-treated stressed mice (Figure 7). Similar to the mice exposed to stress, PPARα mRNA significantly increased in the CORT-treated mice (Figure 7A). Moreover, pretreatment with metyrapone, which fully prevented the stress-induced increase in CORT levels, totally abolished PPARα upregulation induced by stress (Figure 7B). We also studied how CORT treatment affected the expression of the intestinal cholesterol transporters NPC1L1, ABCG5/8, and ABCA1. Comparison of CORT-treated and vehicle-treated groups indicated that CORT administration did not influence the gene expression of NPC1L1, but it upregulated the expression of transporters thought to be directly (ABCG5) or indirectly (ABCA1) involved in cholesterol absorption11 (Figure 7A). However, exposure to stress led, again, to specific downregulation of NPC1L1, whereas inhibition of CORT synthesis by metyrapone abolished this effect (Figure 7B). Because treatment with CORT turned out to be a suitable pharmacological surrogate of the stress effect, we analyzed NPC1L1 protein levels in CORT-treated mice. Despite no apparent effect on NPC1L1 expression at the gene level (Figure 7A), the intestinal NPC1L1 protein levels decreased in the CORT-treated mice (Online Figure XI). These data support the role of CORT as an important mediator in the stress-induced modulation of intestinal PPARα and NPC1L1 expression.

Given that the administration of exogenous CORT induced upregulation of ABCG5 and ABCA1, which are not PPARα target genes, and that intestinal ABCA1 expression has been found to be induced via a PPARα mechanism that involves LXRα,35 we directly investigated whether the stress-mediated response also involved LXRα activation. To this end, we determined the M-RCT rate in PPARα KO and LXRα KO mice exposed to stress (Figure 8). Of note, although the HPA axis is not dysfunctional in these mice, as shown by the significant increase in circulating CORT in response to stress (Figure 8A and 8B), no stimulatory effect was found on the M-RCT rate, which actually tended to decrease (Figure 8C) or remained unchanged (Figure 8D) when compared with controls. These results indicated that mere elevation of CORT by stress was not sufficient to promote M-RCT, but required competent PPARα and LXRα pathways.

Discussion

In mammals, the various physiological responses to stress are mediated by stimulation of the HPA axis, which results in the secretion of glucocorticoids from the adrenal cortex. In the present study, we disclosed a novel mechanism by which a single acute period of stress accelerated M-RCT primarily by impairing the cholesterol absorption function of the intestine without affecting hepatic or intestinal cholesterol synthesis. Of note,
the stress-dependent increase in the M-RCT rate was maintained in mice exposed to stress episodes for at least 7 days. Importantly, we demonstrated that mere elevation of circulating CORT in nonstressed mice triggered such RCT response. Moreover, PPARα was identified as a transcription factor that downregulated the intestinal expression of the cholesterol transporter NPC1L1, known to provide a key pathway for cholesterol absorption in the enterocyte. The prevention of intestinal reabsorption of cholesterol and the concomitant interruption of enterohepatic circulation of cholesterol induced by stress increased the net transfer of macrophage-derived cholesterol into the intestinal lumen and feces, and ultimately facilitates removal of cholesterol from the entire body. To the best of our knowledge, this is the first indication of the effect of psychological stress on intestinal genes involved in RCT regulation.

PPARs and LXRαs are major regulators of intestinal cholesterol homeostasis via transcription of various target genes in the enterocyte. PPARα activation in mice has been found to decrease intestinal expression of NPC1L1 and to promote RCT. In fact, PPARα also was shown to stimulate a non-biliary transintestinal cholesterol efflux pathway that contributes to the total fecal neutral sterol excretion without involving NPC1L1. In contrast, in the present model of acute stress, intestinal upregulation of PPARα, but not of PPARδ, resulted in NPC1L1 repression, thus impairing the intestinal reabsorption of macrophage-derived cholesterol. This was confirmed by the lack of a stress effect on the M-RCT rate and onNPC1L1 expression (Online Figure XI) in PPARα KO mice. In line with our findings, fenofibrate-induced PPARα activation has been found to decrease the expression of NPC1L1 in the small intestine of mice, both at the mRNA and protein levels. Interestingly, intestinal NPC1L1 also is a LXRα target gene, both in mice in vivo and in a human enterocyte cell line in vitro. In addition, it was recently found that intestinal-specific, but not hepatic-specific, LXRα activation leads to decreased intestinal cholesterol absorption, thereby increasing RCT in vivo. Moreover, chronic administration of a synthetic LXR agonist to mice has significantly increased PPARα mRNA levels in the small intestine, but not in the liver. Collectively, these studies support the notion that the small intestine exerts an important regulatory role in the RCT pathway, and strongly suggest the involvement of a complex transcriptional cross-talk between PPARα and LXRα in determining intestinal cholesterol excretion in response to stress.

In contrast to its sharp effect on the intestinal function, exposure to acute stress moderately increased the hepatic uptake of HDL, whereas the cholesterol efflux capacity of serum remained unchanged. Interestingly, neural circuits recently have been found to regulate peripheral cholesterol metabolism in mice, suggesting that central melanocortin signaling up-regulates the hepatic expression of SR-BI mRNA and, thus, decreases HDL-C levels. Because melanocortin has been found to be involved in HPA activation during acute restraint stress, it is plausible that neural-derived signals had induced the overexpression of SR-BI protein also in the present stress model (Online Figure XIII), leading to decreased serum HDL-cholesterol levels. In line with our data, hepatic expression of SR-BI has been shown to be a positive regulator of M-RCT in mice, and it also has been found to increase in mice exposed to social stress. Regarding cholesterol efflux, a complex signaling transduction network among PPARs and LXR has been found to increase ABCA1 mRNA expression in THP-1 cells and mouse fibroblasts. More recently, PPARα agonism in mice was found to trigger a coordinated PPARα/LXR macrophage pathway that enhanced M-RCT by promoting ABCA1/G1 expression and cholesterol efflux in a fashion that depended on apoA-I synthesis. From the present data, it appears that although incubation of macrophages for 3 hours with CORT led to LXRα gene upregulation, PPARα upregulation also was required to induce ABCA1/G1 cholesterol transporter overexpression, at least during a 3-hour incubation period. Furthermore, the ABCA1 gene is selectively repressed by dexamethasone binding to macrophage glucocorticoid receptors, a mechanism that also might apply to CORT. These findings, in addition to the maintenance of basal levels of apoA-I in serum during stress (Online Table I), are compatible with the lack of effect of stress on the efflux machinery efficiency.

Similar to PPARα upregulation induced by glucocorticoids in primary rat hepatocyte cultures and in the liver of rats exposed to restraint stress for 4 hours, in our mouse model, the stress-dependent increase in circulating CORT increased hepatic PPARα expression. More importantly, the present results uncovered a direct link between circulating CORT and PPARα expression in the small intestine during acute stress, with the main effect being a stress-dependent inhibition of intestinal NPC1L1 expression leading to impaired cholesterol absorption. PPARα also has been found to modulate the anti-inflammatory

### Figure 8. Functional peroxisome proliferator–activated receptor (PPARα) and liver X receptor (LXRα) are required for stress-induced acceleration of macrophage-reverse cholesterol transport. PPARα knockout (KO) and LXRα KO mice received intraperitoneal injection of [3H]cholesterol-loaded J774 macrophages immediately before a 3-hour period of physical restraint (stress group). In parallel, other groups of mice were allowed to remain in their cages for a period of 3 hours after the macrophage injection (control group). After the 3-hour period, mice were euthanized to evaluate (A and B) serum CORT and (C and D) the macrophage-derived [3H]cholesterol radioactivity in the contents collected from the small intestinal lumen ([3H]cholesterol+[3H]bile acids), as described in Figure 1. N=6 mice/group. *P<0.05.
effect of glucocorticoids in inflammatory lung and bowel models in mice. Of note, the distinct upregulation of ABCG5 and ABCA1 induced by exogenous CORT suggested the involvement of PPARα-independent regulatory pathways. Particularly, it appeared that high levels of CORT, most likely via a coordinated effect of PPARα and LXRα, exerted redundant effects on the enterocyte net cholesterol balance (ie, by decreasing NPC1L1 gene expression levels and upregulating ABC transporters). In fact, ABCG5/G8 gene expression is stimulated in mice by the LXR agonist T0901317, resulting in an induction of M-RCT. Although the role of intestinal ABCA1 in the efflux of cholesterol from the enterocyte back to the intestinal lumen is controversial, an inverse correlation has been found between intestinal ABCA1 mRNA content and the amount of cholesterol absorbed in mice. An inhibitory effect on cholesterol absorption associated with ABCA1 upregulation was demonstrated to be mediated by LXR or via a PPARα mechanism that involves LXR. Moreover, a specific and independent role of intestinal LXR activation in M-RCT promotion via upregulation of the intestinal ABC transporters A1, G5, and G8 was recently demonstrated. Altogether, our experimental results converged to emphasize the role of CORT in RCT stimulation by a potential mechanism that occurs via a coordinated downstream signaling cascade that mainly targeted the intestine and most likely involved both PPARα and LXRα. Yet, because NPC1L1 is the critical protein for the uptake of cholesterol across the plasma membrane of the enterocyte, the potential significance of the ABC pathways modulating RCT during induction of stress seems to be less relevant. In addition, PPARα appears to cooperate with the HPA axis in regulating energy homeostasis and the immune response via an intricate network of target genes involved in CORT metabolism, whereas LXR influences CORT synthesis by the adrenal glands, which may be particularly important during chronic stress. These data evidence the complex cross-talk existing among PPARα, LXα, and CORT during both acute and chronic stress.

Importantly, although acute restraint stress in rodents is known to modify the intestinal transit time, it has been found to increase colonic motility but not the transit time in the upper part of the intestine, where most of cholesterol is absorbed. Such an increase in colonic motility is only a temporary effect of stress, and the normal flow is recovered after chronic stress. Because the motility of the small intestine was not affected by stress (Online Figure VII) and this intestinal segment was the end point location where M-RCT was measured, it follows that our data were not affected by a change in intestinal motility. Moreover, we demonstrated that M-RCT was accelerated in mice exposed either to a single acute period of stress or whether it is a mere bystander mechanism resulting from a more general effect of increasing CORT and PPARα upregulation in the liver and the intestine, two organs critical in body cholesterol homeostasis.

Acknowledgments

The authors thank Maria Arraño de Kivikko for their excellent technical assistance.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- Stress in humans is considered a risk factor of atherosclerosis, but its effects on experimental atherogenesis are model dependent.
- Reverse cholesterol transport (RCT) is a multistep pathway for elimination of cholesterol in feces, but only the RCT fraction that originates in macrophages (macrophage-RCT) is relevant for atherosclerosis.
- Peroxisome proliferator–activated receptors and liver X receptors regulate intestinal cholesterol homeostasis via several target genes, particularly the gene encoding Niemann-Pick C1-like 1 protein, the major transporter facilitating cholesterol influx into the enterocyte.

**What New Information Does This Article Contribute?**

- Restraint stress in mice, a model of psychological stress in humans, accelerates the rate of macrophage-RCT by suppressing cholesterol absorption in small intestine.

- Administration of corticosterone, the stress hormone in rodents, to nonstressed mice upregulates the peroxisome proliferator–activated receptors-α gene and downregulates Niemann-Pick C1-like 1 protein in the small intestine, and fully reproduced the effect of stress on macrophage-RCT.

- The mechanism of the stress-induced RCT response of the intestine appears to involve a complex cross-talk among corticosterone, peroxisome proliferator–activated receptor (PPAR)α, and liver X receptor (LXR)α.

Stress constitutes a multifaceted body reaction, and short episodes of stress induce physiological responses that support survival. RCT is critical for the maintenance of body cholesterol balance, and increasing RCT rate is thought to reduce the risk of coronary artery disease. Here, we demonstrate that a single acute 3-hour session of restraint stress in mice accelerates the rate of macrophage-RCT. Similarly, exposure to short episodes of stress for up to 7 days also increased macrophage-RCT. Stress significantly decreased cholesterol absorption in the small intestine. The present data suggest that elevated levels of circulating corticosterone triggered the intestinal response to stress. These results indicate that acute episodes of stress increase the ability of the body to eliminate cholesterol and uncover a novel functional connection between the hypothalamic-pituitary-adrenal axis and RCT. These findings may open up new avenues to investigate the potential effects of other glucocorticoids and their pharmacologically active derivatives on cholesterol absorption. The significance of these findings in humans exposed to acute or chronic stress needs to be investigated.
Acute Psychological Stress Accelerates Reverse Cholesterol Transport via Corticosterone-Dependent Inhibition of Intestinal Cholesterol Absorption

Reija Silvennoinen, Joan Carles Escola-Gil, Josep Julve, Noemi Rotllan, Gemma Llaverias, Jari Metso, Annabel F. Valledor, Jianming He, Liqing Yu, Matti Jauhiainen, Francisco Blanco-Vaca, Petri T. Kovanen and Miriam Lee-Rueckert

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SUPPLEMENTAL MATERIAL

Detailed Methods

Animals
C57BL/6J mice were from Harlan Laboratories (Netherlands), human apoA-II transgenic mice (line 11.1) were created as previously described, PPARα-KO mice were obtained from Jackson Laboratory, and LXR-KO mice were kindly donated by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX). Human apoA-II transgenic mice, PPARα-KO mice (http://jaxmice.jax.org/. Stock Number: 008154), and LXR-KO were backcrossed for 10 generations onto the C57BL/6J background. The mice (females, 10 to 13 weeks old) were housed 5 per cage under controlled conditions for light/dark cycle (11/13 h), temperature, and humidity. The animals were kept in the animal facility for at least 1 week before the experiments and were fed a standard chow diet. The experiments were conducted in conformity with Finnish regulations, and the protocols were approved by the National Animal Experiment Board.

Physical restraint stress procedure
To promote acute psychological stress, mice were exposed to a single physical restraint stress period by placing them for 3 h into individual, clear, well-ventilated restraint cylinders (Harvard Apparatus, Cambridge, MA). The restraint procedure was applied at room temperature and between 11:00 am and 3:00 pm to prevent circadian rhythm variations. In some experiments mice were exposed during the light cycle either to 3 sessions of 3-h stress within 24 h or to 1-2 sessions of 2-h stress/day for periods of 5-7 days. Access to food or water was not allowed during the 3-h stress period. Non-stressed (control) mice stayed in their cages in an adjacent animal room and were deprived of food and water during the same time period in which the experimental mice were subjected to stress. After 3 h, both stressed and control mice were sacrificed by isoflurane inhalation. The acute restraint stress procedure described did not induce any significant difference in body weight (not shown).

Analysis of serum
Corticosterone (CORT) and corticotropin releasing factor (CRF) concentrations in serum were determined with ELISA kits (IBL International, Hamburg, Germany, and NovaTeinBio, Cambridge, MA, USA, respectively). Total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured enzymatically with commercial kits (Roche Diagnostics, Basel, Switzerland), and mouse apoA-I was determined by ELISA. Serum was fractionated by FPLC size-exclusion chromatography on a Superose-6 column, and the lipoprotein elution profile was determined by measuring cholesterol and phospholipids in the eluted fractions. Pools of sera were adjusted to a density of 1.21 g/ml, and HDL size was determined by non-denaturing-PAGGE in the isolated lipoprotein fraction. The ability of serum to generate preβ-HDL was evaluated by 2-dimensional immunoelectrophoresis after incubating the serum for 6 h at 37°C with an LCAT inhibitor.

Evaluation of M-RCT
An assay for the in vivo short-time measurement of M-RCT was applied within the 3-h stress period. In brief, [3H]cholesterol-labeled and acetylated-LDL cholesterol-loaded J774 mouse macrophages (1-2x10⁶ cells, 1-2x10⁶ cpm in 0.4 mL of saline) were intraperitoneally (i.p.) injected. Mice were exsanguinated by cardiac puncture under terminal isoflurane anesthesia at 3 h of macrophage injection, and livers and intestines were removed. [3H]cholesterol radioactivity in serum HDL was determined by liquid scintillation counting (LSC) after precipitation of apoB-lipoproteins. The luminal contents of the small and large intestines were collected, and liver and fecal lipids were extracted with isopropyl alcohol-hexane. The lipid layers were evaporated and [3H]cholesterol was counted by LSC. The 3H-radioactivity
in fecal bile acids was determined in the remaining aqueous phase. The amount of $^3$H-radioactivity present in each anatomically defined body compartment was expressed as % of the injected dose. To evaluate the potential effect of the type of cholesterol-donor cell on the RCT assay, the experiments were repeated using J774 macrophages, murine macrophage-like P338D1 cells, or mouse primary peritoneal macrophages. To further disclose any effect attributable to the specific body site where the cells were injected, J774 cells were injected either into the peritoneal cavity or subcutaneously (s.c.) in the scapular region. M-RCT was also measured in mice exposed to repeat sessions of stress during 1 day by evaluating the radioactivity in feces collected within 24 h.

**Measurement of $^3$H/cholesterol efflux from cultured mouse macrophage foam cells**

Cholesterol efflux promoted by mouse serum was evaluated from $^3$H/cholesterol-labeled J774 macrophages prepared as for the M-RCT assay and from C57BL/6J mouse peritoneal macrophages loaded with $^3$H/cholesterol-ester-acetyl-LDL. The cells were incubated for 3 h in DMEM containing mouse serum (2.5%) in the absence of presence of added CORT (50 and 250 ng/mL). The media and the cell lysates were analyzed for $^3$H-radioactivity by LSC. Cholesterol efflux (%) was calculated as 
\[ \frac{\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}}}{\text{dpm}_{\text{cells}}} \times 100 \]

**Hepatic uptake of $^3$H/cholesterol oleate HDL**

HDL (d =1.063-1.21 g/mL) was isolated by differential ultracentrifugation from pools of sera derived from stressed and control mice. HDL preparations were labeled with cholesteryl oleate (PerkinElmer, Waltham MA, USA) as previously described. The labeled HDL preparations were injected intravenously (i.v.) via the tail vein (~80 µg HDL cholesterol/mouse, ~1x10^6 cpm/mouse). Mice that received HDL from stressed mice were exposed to stress, and mice that received HDL derived from control mice stayed in their cages. Blood samples were drawn at 2 min and 3 h, and livers were collected at 3 h after the injection. Livers were collected and subjected to lipid extraction with isopropyl alcohol-hexane. $^3$H-radioactivity was determined in sera and in the liver lipid extracts by LSC, and expressed as % of injected dose. In order to determine the FCR, the cholesteryl oleate-HDL uptake experiment was repeated by exposing mice to stress for 3 consecutive 3-h periods within 24 h. Plasma decay curves for the tracer were normalized to radioactivity at the initial 2-min time point after tracer injection. FCRs were calculated from the area under the plasma disappearance curves fitted to a 2-compartment model, using Prism software.

**Hepatic and intestinal cholesterol synthesis**

 Determination of in vivo cholesterol synthesis was performed in control and stressed mice 3 h after i.p. injection of 10 mCi/kg of $^3$H2O (PerkinElmer). The rate of incorporation of tritium from $^3$H2O into cholesterol in the liver and the small intestine was then determined as described.

**Intestinal absorption of cholesterol**

The efficiency of intestinal cholesterol absorption was determined by a dual isotope ratio method using $^3$Hβ-sitostanol as an internal standard. Briefly, mice received a gastric bolus of 150 µl olive oil containing 1 µCi of $^{14}$C/cholesterol together with 2 µCi of $^3$Hβ-sitostanol (both from American Radiolabeled Chemicals, St. Louis, MO). Immediately after the gavage, mice were either exposed to stress or returned to their cages. The stomachs, intestinal contents, and feces were collected after 3 h, and $^{14}$C and $^3$H radioactivities were determined in the lipid extracts.

**Gene expression of cholesterol transporters by real time-quantitative PCR**

Total RNA was isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA) from J774 cells, livers, and small intestine pools made with equal contributions of the duodenal, jejunal, and ileal tissue sections. RNA samples were repurified, checked for integrity by agarose gel electrophoresis and reverse-transcribed with Oligo(dT) using M-MLV Reverse Transcriptase, RNase H
Minus, Point Mutant to generate cDNA. Predesigned validated primers (Assays-on-Demand, Applied Biosystems, Foster City, CA) were used with Taqman probes. Quantitative RT-PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). All analyses were performed in duplicate, and relative RNA levels were determined using GAPDH as an internal control.

**Western blotting**

Frozen livers were homogenized with mortar and pestle and suspended in modified radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X) with protease inhibitor cocktail (Sigma). Small intestines were flushed once with cold PBS and cut into pieces with equal contributions of the duodenal, jejunal, and ileal tissue sections. The sections were homogenized in a 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM MgCl₂, 0.25 M sucrose and protease inhibitor cocktail (Sigma). From the liver and small intestinal lysates, 75 µg or 100 µg of protein per lane was fractionated on 10 % or 6.5 % SDS-PAGE gel, respectively, and transferred onto nitrocellulose membrane. Following protein transfer, the membrane was blocked and then immunoblotted with polyclonal rabbit anti-mouse SR-BI antibody (Novus Biologicals, Littleton, CO) or with polyclonal rabbit anti-mouse NPC1L1 antiserum. Bound primary antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG (Cell signaling technology, Danvers, MA) and enhanced chemiluminescence (ELC plus, Amershams International). After stripping the membranes, beta-actin (for liver) or GADPH (for small intestine) were immunoblotted as loading controls. Optical density of bands was quantified with ImageJ software (National Institute of Health, USA).

**Pharmacological treatments**

Mice were fed for 10 days with a chow diet supplemented with ezetimibe (0.005% w/w, Teklad, Harlan Laboratories) or PPARδ agonist GW0742 (0.01% w/w, Cayman chemicals, MI, USA) and then exposed to stress for 3 h. In another set of experiments, mice received 2 subcutaneous (s.c.) doses of 5 mg/kg CORT (Sigma Aldrich, St. Louis, MO, USA) or vehicle (1:1 DMSO-saline) immediately before and at 1.5 h after the i.p. injection of the [³H]cholesterol-loaded macrophages, respectively. Other groups of mice were i.p. injected with metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma) (100 mg/kg) to inhibit the endogenous synthesis of CORT or with vehicle (10% DMSO-saline) 90 min before the start of the stress period.

**Statistical analysis**

Unpaired Student’s t-test and non-parametric Mann-Whitney U test were used to compare data between groups. A Pearson correlation was used to analyze CORT and RCT-paired data from mice under the various treatments. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used for all statistical analyses. A P value <0.05 was considered statistically significant. All data are expressed as the mean ± SEM.
Supplemental Reference List


Supplemental Figures and Figure legends

Supplemental Figure I. Lipid profile of serum lipoproteins in mice exposed to stress
C57Bl/6J mice were either exposed to restraint stress for 3 h (stress) or allowed to stay in their cages for the same period without food or water (control). Serum was obtained from blood collected immediately after the 3-h experimental period, and representative pools of serum (100 μl each, 3 mice/group) were applied to superose 6HR size-exclusion chromatography column. The eluted fractions were analyzed for (A) cholesterol and (B) choline-containing phospholipids.
Supplemental Figure II. Time-course of serum cholesterol, apoA-I, and mediators of the HPA axis during restraint stress up to 3 h
C57Bl/6J mice were exposed to restraint stress for 0 (basal), 10, 30, 60, 120, and 180 min. Serum was obtained from blood collected immediately after the indicated time periods. (A) Pools of sera were adjusted to a density of 1.21 g/ml, and HDL size was determined by ND-PAGE of the isolated lipoprotein fraction. Individual serum samples were analyzed for (B) total cholesterol (TC) and apoA-I, (C) CRF, and (D) CORT concentrations. N= 3 mice/group. *P< 0.05, as compared with the basal levels.
Supplemental Figure III. Transfer of macrophage-derived \( ^3 \)H/cholesterol from peritoneal and subcutaneous sites to the small intestine contents

C57Bl/6J mice received i.p. injections of either (A) \([^{3}H]\)cholesterol-labeled macrophages isolated from the peritoneal cavity of control mice (MPM, n= 3 mice/group, \( p=0.038 \)) or (B) \([^{3}H]\)cholesterol-labeled P388D1 macrophages (n= 5 mice/group, \( p=0.049 \)). Immediately after injection of the labeled macrophages mice were either exposed to 3 h of physical restraint (stress group) for a period of 3 h (control group). The macrophage-derived \([^{3}H]\)cholesterol radioactivity was measured 3 h after macrophage injection in the contents collected from the small intestinal lumen. (C) Another group of mice received s.c. injection of \([^{3}H]\)cholesterol-labeled J774 cells in the scapular region, immediately followed by 3 sessions of 3-h stress within 24 h or allowed to remain in their cages (n= 3-4 mice/group, \( p= 0.044 \)). Lipids were extracted from the small intestinal lumen contents collected after the 24 h period, radioactivity was measured, and expressed as % of the injected dose. *\( P< 0.05 \).
Supplemental Figure IV. Cholesterol efflux from mouse macrophages stimulated by serum from mice exposed to stress

$[^3]$H]Cholesteryl ester-labeled J774 foam cells were incubated in DMEM media containing mouse serum in the presence of 0, 50, and 250 ng/ml CORT. A sera pool derived from 4 mice was added to media at 2.5% (v/v) final concentration. After 3 h of incubation, macrophage cholesterol efflux was determined and expressed as $\text{dpm}_{\text{medium}} / (\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}}) \times 100$. 

![Graph showing cholesterol efflux from J774/3 h (%) vs. Corticosterone (ng/ml)](image-url)
Supplemental Figure V. Hepatic uptake of HDL cholesterol after repeat sessions of stress within 24 h

HDL isolated from control and stressed mice were labeled with [3H]cholesteryl oleate. HDL uptake by the liver was evaluated after i.v. injection of the autologous radiolabeled HDL preparations, as described in Methods, to control and stress mice (n= 4 mice/group). To measure the HDL [3H]cholesteryl oleate clearance (arbitrary units, AU), blood samples were drawn from the tail vein at 2 min, 3 h, 9 h, and 24 h. The % of injected dose at 2 min was 57±5 and 51±6 % in control and stress mice, respectively. (A) Serum decay curves for the tracer were normalized to radioactivity at the initial 2-min time point after tracer injection. FCRs were calculated from the area under the plasma disappearance curves fitted to a two-compartment model (inset). (B) At study termination (24 h after injection), livers were collected and lipid were extracted. The [3H]radioactivity in the liver was measured and expressed as % of the injected dose. N= 4 mice/group. *P< 0.05.

![Graph A](image1.png)

![Graph B](image2.png)
**Supplemental Figure VI. De novo synthesis of cholesterol in liver and small intestine in stressed mice**

C57Bl/6J mice were i.p. injected with 10 mCi/kg of $^3$H$_2$O and were either exposed to 3 h of physical restraint (stress group) or allowed to remain in their cages for a period of 3 h (control group). Three hours later the animals were sacrificed, plasma was withdrawn and the indicated tissues were removed, weighed, and saponified. The specific activity of the tritiated water in plasma was calculated for each animal by dividing the $^3$H-radioactivity (dpm/ml plasma) by the mmoles of water/ml plasma (52 mmol/ml plasma, assuming that plasma is 93% water). Specific activities were similar in control and stressed mice (158,500 ± 11,800 versus 160,200 ± 4,500 dpm/mmol, $P=0.90$). The incorporation of $^3$H$_2$O into cholesterol was determined after lipid extraction of the tissues with petroleum ether. N= 4 mice/group.

![Figure](image_url)
Recovery of $[^3]$H$\beta$-sitostanol along the gastrointestinal tract was evaluated in mice exposed to stress and the control group by calculating the % of the administered dose in mice that received a mixture of $[^{14}]$Ccholesterol and $[^3]$H$\beta$-sitostanol by gavage, as described in Figure 2C. N= 6 mice/group. **P<0.01.
Supplemental Figure VIII. Contents of cholesterol in adrenal glands isolated from human apoA-II transgenic and the wild-type mice

Adrenal glands were dissected from the human apoA-II transgenic (hApoA-II Tg) and the C57Bl/6J (wild-type) mice. Adipose tissue-free adrenal glands were extracted with isopropyl alcohol-hexane, and cholesteryl esters and unesterified cholesterol were quantified in the lipid extracts and expressed per weight of tissue. N= 8-9 mice/group. ***P<0.001.
**Supplemental Figure IX. Loss of the stress effect on M-RCT in mice with deficient corticosterone production**

Human apoA-II transgenic (hApoA-II Tg) mice received intraperitoneal \[^3\text{H}\]cholesterol-loaded J774 macrophages and were immediately exposed to physical restraint (stress) or were allowed to remain in their cages (control) for a period of 3 h. After the 3-h period, all mice were sacrificed to evaluate (A) serum CORT and (B) the macrophage-derived \[^3\text{H}\]cholesterol radioactivity in the small intestinal lumen contents (\[^3\text{H}\]cholesterol+\[^3\text{H}\]bile acids). N= 6 mice/group.
Supplemental Figure X. Correlation between circulating corticosterone and M-RCT
Pearson correlation test was applied to correlate serum CORT with the rate of M-RCT (A) to the small intestinal contents as described in Figure 1 and (B) to feces collected within 24 h, as described before. Paired values in panel A are derived from all mice in which CORT and 3 h-RCT were evaluated, i.e., whether the excess of circulating CORT was achieved by exogenous CORT administration or by a stimulation of a physiological (stress-dependent) CORT synthesis. Genetically modified mice and ezetimibe- and GW0742-treated mice were excluded from this analysis. Paired values in panel B are derived from C57Bl/6J mice exposed to either 3 sessions of 3-h stress within 1 day or 1-2 sessions of 2-h stress/day for periods of up to 5-7 days. All stress sessions were applied in the light phase of the day. In all groups of stressed mice, M-RCT significantly increased in comparison with the non-stressed corresponding control mice. Fecal [\(^3\)H]cholesterol tracer levels of mice exposed to stress sessions (C) within 1 day or (D) for periods of 5-7 days. Feces were collected continuously from 0 to 24 h after i.p. injection of the [\(^3\)H]cholesterol labeled macrophages. *P<0.05; **P<0.01.
**Supplemental Figure XI. Intestinal NPC1L1 protein expression in control and corticosterone-treated mice**

Small intestines were collected from mice that had received 2 s.c. doses of CORT (5 mg/kg) or vehicle (1:1 DMSO-saline) as described in **Figure 5**. (A) NPC1L1 protein expression was analyzed by Western blotting. Samples of small intestine from wild-type (WT) and NPC1L1-KO mice were used as positive and negative controls, respectively, and GAPDH was immunoblotted as a loading control. (B) Protein expression was quantified by optical density analysis performed with ImageJ software. Results are expressed in arbitrary units (AU). N=3 mice/group. *P<0.05.
Supplemental Figure XII. Gene expression of transcription factors and cholesterol transporters in the small intestine of PPARα-KO mice exposed to stress

The relative mRNA expression of transcription factors and cholesterol transporters was analyzed by quantitative RT-PCR in the small intestine of PPARα-KO mice, as described in Figure 3. These mice were used also in the M-RCT experiment shown in Figure 8. N= 6 mice/group.
Supplemental Figure XIII. Hepatic SR-BI protein expression in control and stressed mice
(A) SR-BI protein was measured in liver homogenates of control and stressed mice by Western blotting. After stripping of the membrane, β-actin was immunoblotted as a loading control. (B) Protein expression was quantified by optical density analysis performed with ImageJ software. Results are expressed in arbitrary units (AU). N=4-5 mice/group. **P<0.01.
Supplemental Tables

**Supplemental Table I.** Effect of stress on the serum levels of corticosterone and other serum parameters related to lipoprotein metabolism in C57Bl/6J mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Stress</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>122 ± 16</td>
<td>593 ± 31***</td>
<td>11</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>2.10 ± 0.11</td>
<td>1.80 ± 0.10*</td>
<td>11</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.51 ± 0.11</td>
<td>1.19 ± 0.07*</td>
<td>11</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.51 ± 0.03</td>
<td>0.46 ± 0.06</td>
<td>11</td>
</tr>
<tr>
<td>ApoA-I (g/l)</td>
<td>1.04 ± 0.04</td>
<td>1.06 ± 0.03</td>
<td>6</td>
</tr>
<tr>
<td>LCAT (nmol/ml/h)</td>
<td>18.8 ± 2.18</td>
<td>15.5 ± 1.67</td>
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</tr>
<tr>
<td>Preβ-HDL generation (%)</td>
<td>6.7 ± 0.66</td>
<td>6.2 ± 1.03</td>
<td>6</td>
</tr>
</tbody>
</table>

Mice were either placed in restrainers (stress) or were allowed to remain in their cages (control) for 3 h. Sera were obtained from blood collected immediately after the 3-h period. N= number of mice/group. *P< 0.05; ***P< 0.001.
**Supplemental Table II.** Effect of corticosterone administration on the serum levels of corticosterone and lipids in C57Bl/6J non-stressed mice

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>CORT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticotropin releasing factor (ng/ml)</td>
<td>25.9 ± 2.34</td>
<td>12.3 ± 2.40***</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>225 ± 21</td>
<td>578 ± 84***</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>2.1 ± 0.12</td>
<td>2.1 ± 0.08</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.0 ± 0.04</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9 ± 0.13</td>
<td>1.0 ± 0.12</td>
</tr>
</tbody>
</table>

Wild-type C57Bl/6J mice received s.c. doses of corticosterone (5 mg/kg) or vehicle (1:1 DMSO-saline) and were allowed to stay in their cages. After 1.5 h, mice received a second s.c. dose of corticosterone (5 mg/kg) or vehicle and were returned to their cages. Three hours after the initial injection, all mice were sacrificed, blood was collected, and serum analysis was performed. N= 6 mice/group. ***P< 0.001.
**Supplemental Table III.** Effect of stress on serum lipids of human apoA-II transgenic mice

<table>
<thead>
<tr>
<th>Lipid (mmol/l)</th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>1.4 ± 0.15</td>
<td>1.4 ± 0.14</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.7 ± 0.12</td>
<td>0.8 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.5 ± 0.11</td>
<td>0.9 ± 0.08**</td>
</tr>
</tbody>
</table>

Human apoA-II transgenic mice were either placed in restrainers (stress) or were allowed to remain in their cages (control) for 3 h. Sera were obtained from blood collected immediately after the 3-h period. N= 5 mice/group. **P< 0.01.