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Rapid Decrease and Subsequent Increase in the Serum Triglycerides Accompanied by *CD36* Transcript Increase in an Acute Stress Mice Model

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Abstract

In this article, we report the changes in serum triglyceride (TG) levels that occurred during repeated tail blood sampling using a mouse restrainer. We used three groups of mice, namely, "PBS-restrained" "PBS-unrestrained" and "mock-restrained". The mice in the PBS-restrained and PBS-unrestrained groups were intraperitoneally (i.p.) injected with 100 mL PBS and tail blood sampling was performed at 1, 5, 8, 24, and 48 h after i.p. injection. For the mock-restrained group, no i.p. injection was performed whereas the subsequent tail blood sampling was similarly performed. During the tail blood sampling, the mice of the two "restrained" groups were placed inside the restrainer designed from an open-ended 50 mL conical tube. The blood from the mice in the PBS-unrestrained group mice was sampled from the tail held by the operator's hands while being allowed to move on a stage. Strikingly, in all of the three groups, the serum TG level initially decreased to remarkably low levels (approximately 30 mg/dL) after several blood samplings were performed over 8 h. This decrease was followed by a 2 - 3-fold increase in the levels relative to that in the control mice in the subsequent 24 - 48 h time period. We concluded that the acute stress associated with blood sampling caused alterations in TG levels. Serum levels of free fatty acid showed only modest changes. Changes in TG levels were not associated with serum corticosterone levels but with a dramatic increase in *CD36* transcript levels in the liver. The relevance of this finding to the previously reported release of lipoprotein lipase (LPL) from white fatty tissue into the plasma during acute stress is also discussed.

Keywords

Acute Stress, Lipoprotein Lipase, CD36, Plasma Triglyceride

1. Introduction

Various stimulations, including acute stress, cause rapid changes in lipid metabolism, leading to changes in plasma triglyceride (TG) levels [1] [2] [3]. Besides its regulatory role in regulating the metabolism, assessing the extent to which acute stress confounds the analyses of metabolic parameters is of paramount importance, given that blood sampling, such as that from the tail, is a procedure often used with rodent models to analyze systemic metabolism.

In our experiments, we examined the effects of Toll-like receptor (TLR) ligands on metabolism [4]. We incidentally observed that even the control mice group (injected with phosphate-buffered saline (PBS)) showed remarkable decreases in serum TG levels (Seki, unpublished result). Hence, in the present study, we analyzed the serum levels of TG, free fatty acids (FFAs), glucose, and corticosterone during acute stress associated with blood sampling using a mouse restrainer. Memon *et al.* reported that fatty acid translocase/CD36 is upregulated in the liver in the endotoxemia model [5]. Therefore, we measured the level of *CD36* transcripts in the liver during the period of rapid TG decrease. The effects of acute stress on the blood lipid profile and hepatic CD36 expression are discussed.

Stress-induced TG level changes have been analyzed in rat [2] and mice [6], yet our present study has unique features. First, the experimental settings used in these preceding studies were aimed solely for imposing stress on animals; specifically, the animals were fixed by taping in both studies [2] [6]. In contrast, we used a tail blood sampling procedure, which is, as far as we know, commonly used in laboratories. Moreover, these studies highlighted decreases of TG whereas we also analyzed the changes after the initial decrease up to two days. Another feature of our study is the measurement of the level of *CD36* transcripts in the liver after imposing the stress.

2. Materials and Methods

Female C57BL/6 mice (7 - 8 weeks old) were provided access to food and water ad libitum and maintained on a 12-h light/dark cycle (lights on at 8 AM). In the first set of experiments, 15 mice were divided into three groups (n = 5 each): "PBS-restrained" "PBS-unrestrained" and "mock-restrained". On the day of the experiment, at 9 AM (0 h), all mice in the PBS-restrained and PBS-unrestrained groups were intraperitoneally (i.p.) injected with 100 mL PBS. In the mock-restrained group, the syringe needle penetrated the peritoneal cavity, but no injection was performed. Blood (50 mL) was sampled from the tail veins of all mice at 1, 5, 8, 24, and 48 h after i.p. injection. The mice were basically kept

in the cage with the exception of the time of blood sampling, when the mice of the two "restrained" groups were placed inside the restrainer designed in-house from a 50 mL conical polypropylene tube by making a hole and placing a porous material that enabled breathing. The blood from the mice in the PBS-unrestrained group was sampled from the tail held by the operator's hands while being allowed to move on a 20 × 20 cm stage made of a rectangular parallelepiped box with a height of 40 cm. Owing to the difficulty in puncturing the tail veins of young mice, a partial cut of the tail vein was performed using a scalpel blade, followed by collecting the blood that appeared on the skin surface into capillary glass tubes. No anesthetics were used for blood sampling.

Serum TG, FFAs, and alanine aminotransferase (ALT) levels and whole blood glucose were measured using Drychem (Fujifilm, Japan) according to the manufacturer's protocol.

Serum corticosterone levels were measured using an ELISA assay (Cayman) following the manufacturer's protocol. Results obtained from the PBS-restrained mice were compared to those obtained for the "restrained" group subjected to repeated blood sampling using the restrainer without the preceding penetration/injection.

An additional four groups of mice were used for measuring the *CD36* transcript in the liver (n = 3 for each of the "control" and "PBS-restrained-8," "PBS-restrained-24," and "PBS-restrained-48"). Mice in the PBS-restrained-8, -24, and -48 groups were sacrificed for liver sampling at 8, 24, and 48 h, respectively, after the treatment, similar to the procedure used for the PBS-restrained group. The mice in the control group were not subjected to any treatment and were sacrificed for liver sampling. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed as follows: The total liver RNA was purified using the NucleoSpin RNA kit (Macherey-Nagel, Germany). After reverse transcription using random primers, cDNA was subjected to qPCR analysis. Hypoxanthine phosphoribosyl transferase (*HPRT*) transcript was used as the internal standard. The CD36/HRPT transcript ratio was measured using TB Green® Premix Ex Taq™ II (Takara Bio, Japan) following the manufacturer's protocol. The primer sequences (5'-3') were as follows:
CD36 forward, GGCCAAGCTATTGCGACATG;
*CD*36 reverse, CCGAACACAGCGTAGATAGAC;
HPRT forward, TTGTTGTTGGATATGCCCTTGACTA,
HPRT reverse, AGGCAGATGGCCACAGGACTA. The cycling conditions were as follows: 94˚C for 3 min, followed by 40 cycles of 94˚C for 20 s, 65˚C for 20 s, and 72˚C for 15 s.

All experiments were performed using protocols approved by the experimental animal committee of Teikyo University.

3. Results

In all PBS-restrained, PBS-unrestrained, and mock-restrained groups, a rapid

decrease in serum TG was observed 1 - 8 h after the initial injection (*i.e.*, 0 - 7 h after the first blood sampling) (**Figure 1(a)**). In all three groups, the TG level decreased significantly to approximately 30 mg/dL at 8 h, compared to the data at 1 h. In addition, the serum TG level exhibited a rebound later for all three groups, increasing to approximately 2 - 3-fold of the basal level (**Figure 1(b)**). We concluded that these TG level changes were caused by acute stress due to the blood sampling procedure. Regardless of whether the restrainer was used in blood sampling, the acute stress caused by the tail blood sampling procedure can become severe to the extent that it affects the TG levels in the circulation.

Serum ALT activity increased at 5 and 8 h relative to 1 h in the PBS-restrained group (**Figure 1(c)**). A slower increase (*i.e.*, at 8 - 24 h) was observed in the mock-restrained group. While serum ALT increase was not a robust feature, these findings suggest that this increase could occur depending on the degree of acute stress.

The time course of the serum FFAs did not show significant changes for the PBS-unrestrained and the mock-restrained groups, but showed a rapid decrease in the PBS-restrained group (**Figure 1(d)**). However, the blood glucose levels did not show any clear trend (**Figure 1(e)**). Thus, serum TG levels appear to be a highly sensitive marker for the severity of acute stress compared to serum FFA and glucose levels. Serum corticosterone levels of PBS-restrained groups mice also did not show a clear trend (**Table 1**), arguing against the role for corticosterone in TG regulation.

To gain some insights into the mechanism underlying the serum TG decrease, we measured the CD36 transcript level in the liver of the mice sacrificed at 8, 24, and 48 h after handling (i.p. injections and tail blood sampling) in a manner similar to that of mice in the PBS-restrained group. A significant increase (>10,000 fold) was observed at 8 h, and the transcript level remained high until 48 h (**Table 2**). These findings suggest that rapid hydrolysis of TG is at least partly coupled with the rapid uptake of FFA by the liver, causing a modest level of ALT leakage from hepatocytes into circulation.

Table 1. Serum corticosterone levels (ng/mL).

	1 h	5 h	8 h	24 h	48 h
restrained	60 ± 10	48 ± 18	75 ± 21	48 ± 13	46 ± 3
PBS-restrained	75 ± 38	51 ± 14	70 ± 13	48 ± 6	70 ± 18

Table 2. RT-qPCR analysis of *CD36* transcript levels in the liver.

Control	PBS-restrained*		
	8 h	24 h	48 h
1 ± 0.2	11,684 ± 916	15,707 ± 2,821	12,378 ± 2,396

*The *CD*36/*HPRT* ratio relative to that of the control (wild-type) mice that were kept in the cage normally until the experiment. All 8, 24, and 48 h data showed statistical differences ($p < 0.01$) compared to the control mice data.

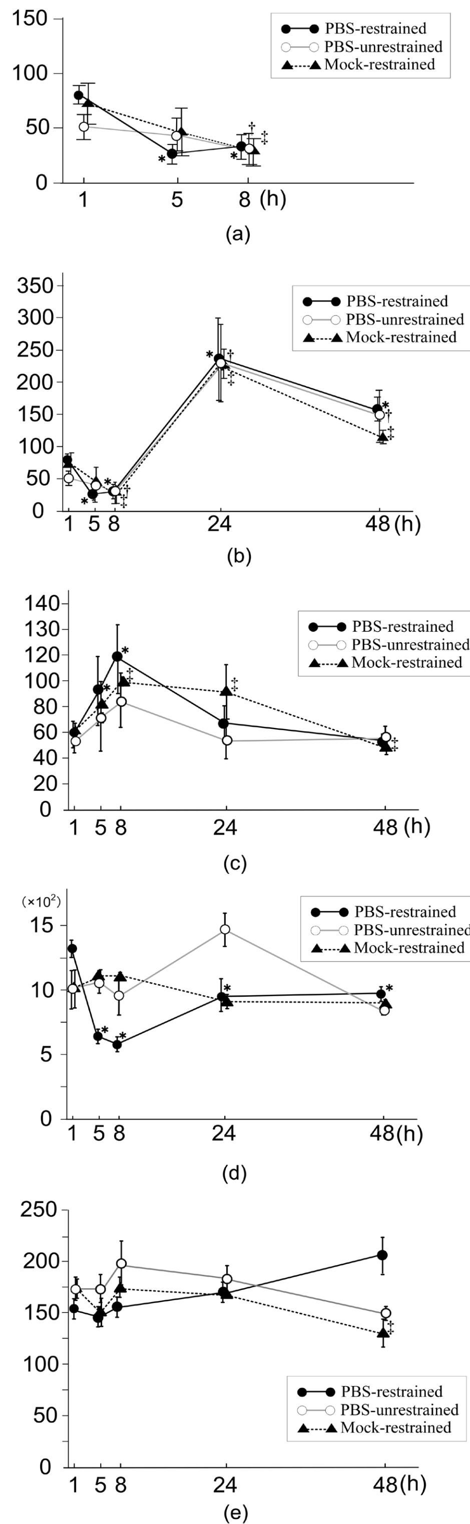

Figure 1. The serum metabolic parameters plotted as a function of the time after the i.p. injection (or mock injection): ((a), (b)) TG (mg/dL), (c) ALT activity (U/L), (d) FFAs (nmol/ml), and (e) glucose (mg/dL). Notably, the tail blood sampling was performed at 1, 5, 8, and 48 h after i.p. The symbols *, †, and ‡ denote the statistical significance ($p < 0.05$) compared to the corresponding 1 h data for the PBS-restrained, PBS-unrestrained, and mock-restrained groups, respectively.

4. Discussion

The present study was motivated by our incidental observation of a rapid change in TG levels in all mice, including PBS-injected control mice (unpublished observation), in an experiment to study the effects of TLR ligands on metabolism [4]. The findings in the present study imply that multiple tail blood samplings in mice without anesthetics can impose acute stress, complicating the studies, particularly TG metabolism. The TG level in the circulation appears to be a sensitive parameter that changes rapidly in response to acute stress and might serve as a superior stress marker compared to blood glucose or corticosterone levels. In contrast, the serum FFA levels did not show significant changes, suggesting rapid uptake of the TG hydrolysis products.

A pronounced increase in hepatic *CD36* transcript was also observed, supporting this suggestion. CD36 is a multi-ligand scavenger receptor belonging to the class B scavenger receptor family. It is expressed in various mammalian tissues/cells, including hematopoietic cells, endothelial cells, enterocytes, adipocytes, and cardiac and skeletal muscle cells [7]. The basal level of CD36 expression in the liver is substantially low. However, it has been shown to increase dramatically by a high-fat diet or by the activation of nuclear receptors [8] [9]. In an early study by Memon *et al.*, an endotoxemia rodent model showed increased *CD36* transcript levels in the liver. To the best of our knowledge, the present study is the first report of *CD36* transcript upregulation due to acute stress.

Acute stress causes a rapid decrease (1 - 10 h) in plasma/serum TG in mice and rats [2] [6]. In the immobilization stress rat model, a remarkable decrease in plasma TG was observed after only 5 min of immobilization stress [10]. Our results confirmed such an effect of stress and revealed that the initial decrease in the TG level is followed by a rapid 2 - 3-fold increase over 24 to 48 h compared to that in the normal control mice. Notably, *CD36* transcripts in the liver showed a dramatic increase. Although quantitative evaluation of CD36-mediated uptake in stress-induced TG changes requires further analysis, it is plausible that CD36 upregulation facilitates the hepatic uptake of FAs (product of hydrolysis) and lipoproteins themselves. As reported in acute stress models, rapid uptake may have caused a modest level of ALT release from the liver into circulation (**Figure 1(c)**). For example, both AST and ALT in the plasma increased after 3 h of immobilization [11]. Our results suggest that upon acute stress, the hepatic uptake of hydrolysis products of TG may rapidly increase, causing mild liver damage. However, corticosterone levels were largely stable, arguing against a significant role of this hormone in TG level changes. The implications of our findings on lipid accumulation and lipotoxicity implicated in nonalcoholic fatty liver disease (NAFLD) remain unclear.

Of note, our findings raise the issue that, in analyses using rodent models, repeated tail blood sampling without anesthetics could serve as a confounding factor through perturbations in lipids metabolism. Our previous experiments

using TLR ligands including LPS in combination with D-galactosamine yielded essentially similar results compared to the previous reports in this area [12]. Therefore, we consider that, in the case of liver injury model using TLRs/D-gal, the repeated blood sampling does not seriously caused diverse results depending on whether an anesthetic is used or not. Nonetheless, our findings call for further attention to the lipids metabolism effects, which could confound the results of experiments in many settings.

Lipoprotein lipase (LPL) plays a major role in rapid TG regulation in circulation under various stresses/challenges. Acute stress can rapidly trigger the release of LPL from the white adipose tissue (WAT) into the blood [2] [13]. A similar release of LPL also occurs in an endotoxemia rat model [3], LPL levels in tissues are flexibly regulated, depending on the fuel requirements in the tissues. However, several questions regarding the LPL remain. First, the mechanism of LPL release from WAT under acute stress is poorly understood. In acute stress, catecholamines are likely to mediate release [14] and increase NO synthesis [15]. However, the link between NO synthesis and release remains unclear. Nitration of the enzyme may be important [16], but the contribution of this process remains to be determined. Second, the occurrence of LPL hydrolysis remains unclear. The rapid decrease in serum TG level suggests that the lipoprotein surface is the primary site for hydrolysis. However, albumin-bound FFAs are not efficient for the activation of peroxisome proliferator-activated receptors (PPAR)-alpha that upregulates CD36 compared to FFAs generated by LPL-hydrolysis [17]. LPL-mediated hydrolysis might occur near the endothelial cells of the sinusoid or even closer to the hepatocyte surface, enabling rapid uptake of FAs immediately after hydrolysis. Third, while LPL is known to function as a tethering molecule independent of its catalytic activity, the importance of this function in the acute stress model is unknown. Further studies are required to understand these issues better.

The mechanism underlying rapid CD36 upregulation in the liver remains largely unknown. Memon *et al.* showed that the *CD36* transcript increases in the liver, whereas it decreases in many other tissues in an endotoxemia hamster model. Our findings support the view that hepatocyte mechanisms dramatically upregulate CD36 expression after various stimuli. Liver X receptor (LXR), pregnane X receptor (PXR), and PPAR-gamma are thought to act synergistically to upregulate CD36 expression [18]. Notably, the regulation of CD36 by LXR and PXR, as well as the regulation of PPARγ by PXR, is liver-specific [8] [18]. However, the mechanism underlying CD36 upregulation during stress or endotoxemia remains largely unknown. Envisaging that LPL directly activates CD36 and facilitates FA uptake is an interesting notion, although direct interaction has not been well documented.

Considering the implications of rapid TG level regulation in infections and inflammation is of interest. High levels of TG-rich lipoproteins in sepsis and endotoxemia have been considered beneficial as they can neutralize LPS, lipoteichoic acid, and other lipidic molecular patterns of microorganisms [1]. Notably,

aggressive encounters have been shown to predispose mice to the toxic effects of bacterial LPS [19]. Social disruption stress has also been shown to enhance the susceptibility of mice to LPS challenge, as measured by pro-inflammatory cytokine expression levels in several organs [20]. Several studies, including ours, have suggested that rapid changes in plasma TG levels play a crucial role in stress-induced susceptibility to endotoxic shock.

Given the beneficial effect of a high level of plasma TG in infection [1], one may ask why TG quickly decreases before it increases later. We hypothesize that rodents may experience acute stress associated with an injury in the wild that increases the chance of entry of microbes. The initial decrease in plasma TG may facilitate the rapid clearance of lipidic molecular patterns immediately after the injury. In contrast, the later increase in TG may help neutralize the lipidic molecular pattern of microorganisms that may have increased shortly after acute stress [1]. Further studies are required to understand the evolutionary implications of the rapid lipid regulation during acute stress.

Finally, given the technological improvements in blood parameter measurements, more future studies may uncover rapid changes in common biomarkers after diverse interventions. Our focus was the effects of stress, but potential impacts of food ingredients may be another interesting research area. For example, a remarkable increase in the white blood cell count and a decrease in the hemoglobin concentration after an intake of common artificial sweetener aspartame has been reported [21]. It is hoped that such studies including ours underscore potential usefulness of common biomarkers in rodent models with a variety of interventions.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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