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On the Impairment of Stress-Induced Changes in Triglyceride Levels via a Sub-Toxic Dose of Unmethylated Cytidine Phosphate Guanosine Oligodinucleotide (a Toll-Like Receptor 9 Ligand)

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Abstract

Changes in lipid metabolism have been implicated in protection against infectious diseases. In the first experiment of this study, we measured clinical lipid parameters in a murine model where the unmethylated cytidine phosphate guanosine (CpG) oligodinucleotide (ODN1826), a Toll-like receptor 9 (TLR9) agonist was administered in combination with D-galactosamine (GalN) that caused relatively liver-specific inflammation and toxicity. In the control mice group injected with phosphate-buffered saline (PBS) (acute psychological stress model associated with blood sampling), the serum triglyceride (TG) levels showed a rapid decrease followed by a rebound at 24 h as we have recently reported. However, such a TG rebound was impaired in the CpG/GalN- and solely CpG-treated groups of mice despite an absence of liver injury based on serum alanine aminotransferase levels in the latter group. Thus, the stress-associated serum TG rebound was abrogated by the injection of a sub-hepatotoxic CpG dose. In the second experiment, we simply measured the hepatic *CD36* and *SACRB1* (the gene for scavenger receptor B1 (SR-B1)) transcripts after the i.p. administration of PBS, CpG or CpG/GalN. There was a remarkable elevation of hepatic *CD36* transcript expression in both the CpG- and CpG/GalN-treated mice at 8 h post-CpG injection whereas the increase in the PBS-treated mice was slower than the former two groups, suggesting that hepatic *CD36* transcript expression is more pronounced in the combined stress models than under psychological stress alone. The individual mice data showed that the increase in

CD36 expression was accompanied by a reduction in *SCARB1* mRNA, showing reciprocal regulation between these two genes. Together with our previously reported findings, these data suggest that, in a murine model combining psychological stress with TLR-triggered hepatic inflammation, the psychological stress facilitates liver uptake of plasma TG (and its components fatty acids), but the subsequent re-esterification and/or release of TG-rich lipoproteins from the liver is impaired due to the concomitant TLR-signaling. We hypothesize that lipid metabolism during acute stress shifts toward an elevated hepatic uptake of lipids due to concomitant TLR signaling, facilitating the clearance of bacterial lipids by the liver.

Keywords

Toll-Like Receptor 9, Cytidine Phosphate Guanosine Oligodinucleotide, Scavenger Receptor B1, Triglyceride, Hepatic Inflammation

1. Introduction

Regulation of lipid metabolism is essential in the control of the systemic metabolism. Lipid transport among organs as well as the energy metabolism of many organs and tissues are regulated by many factors. A number of hormones, including insulin, adipocyte hormones, glucocorticoids, leptin, and thyroid hormones, are well-known players in the regulation. Catecholamines and the sympathetic nervous system also play an important role in the triglyceride (TG) metabolism [1], accounting for the coordination of the diet and the lipid metabolism. Regarding the role of the neuroendocrine system in lipid metabolism, administration of adrenaline and acute stress have been shown to cause an increase in plasma lipoprotein lipase (LPL) activity coinciding with a decrease in white adipose tissue LPL activity [2] [3]. LPL is one of the major regulators of the plasma TG level: the higher plasma LPL activity generally corresponds to the higher activity of the cellular utilization of the fatty acids (FAs) comprising the TG in the plasma [4]. Besides the endocrine systems, several proinflammatory cytokines can produce changes in plasma TG levels [5], implying the interplay between immune system and the lipid metabolism. Although we cannot discuss it in detail here, other factors regulating lipid metabolism include short-chain fatty acids produced by the bacterial flora [6] and vitamins [7].

Recent studies of the innate immunity postulate that immunological stimulations trigger cell responses, where the triggers can be either of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs). The DAMPs are generated in the integrated stress response (ISR), for which heat shock response, unfolded protein response, DNA damage response, and the responses to oxidative stress are the well-studied elements [8] [9]. In immune cells such as macrophages, the signaling pathways triggered by the detection of PAMPs or DAMPs via a wide range of pattern-recognition receptors (PRRs) including Toll-like receptors lead to the activation

of NF-κB, the master regulator of inflammation and innate immune homeostasis. The ISR signaling promotes communication between neighboring cells in local inflammation through DAMPs that serve as communication signals [10]. The activation of NF-kB promotes transcription of a large set of pro-inflammatory genes [11]. Thus, we can use the term "immunological stress" to indicate the cellular and systemic responses to bacterial or viral components with the consideration that aseptic cellular stress responses can also cause similar proinflammatory cytokines production. Immunological stress promotes the elimination and inactivation of pathogens, but, in a broader scope, promotes the protection and recovery of tissues from a wide variety of cellular stresses. Although we do not review in depth here, a number of recent studies focus on the formation of NLRP3 inflammasome, implicating this formation in the crosstalk between inflammation and lipid metabolism regulation. This is of clinical relevance as the formation of NLRP3 inflammasome by DAMPs is considered to have a central role in obesity-induced inflammation, insulin resistance and type 2 diabetes mellitus [12] [13].

Although the experimental studies addressing the systemic metabolism changes upon the immunological stress are relatively few, a wide range of changes in lipid metabolism are known to occur during infection and inflammation [5]. For example, increased serum TG levels and reduced oxidation of FA in multiple organs have been reported in several animal experiments mimicking sepsis [5] [14]. Notably, some of these changes in lipid metabolism during infection and inflammation have shown beneficial effects in experimental systems [5]. For example, high levels of TG-rich lipoproteins in sepsis and endotoxemia have been shown to be beneficial: it is hypothesized that they can help in the neutralization and disposal of microbe-derived lipids such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), PAMPs derived from Gram-negative and Gram-positive bacteria, respectively [5] [15]. Inactivation (detoxification) of LPS and LTA by chylomicrons and lipid-binding proteins (LBPs) has been shown by Vreugdenhil *et al.* [16]. Harris *et al.* showed that chylomicrons can protect rats from endotoxin toxicity, partly by facilitating endotoxin clearance [17], as we consider further in the Discussion section.

Regarding metabolism during systemic inflammation, it has been well-documented that the situation in the liver is different from that in other organs [18]. The administration of LPS, tumor necrosis factor (TNF) or IL-1 suppressed FA oxidation in multiple tissues and caused adipose tissue lipolysis, but enhanced hepatic uptake of FA and re-esterification of FA into TG [18]. Consistent with this, LPS injection caused a reduction in the mRNA levels of FA transport protein (FATP) and CD36 protein (formerly termed FA translocase, or FAT or platelet glycoprotein 4) in multiple organs; however, in the liver, while the *FATP* mRNA decreased, the *CD36* mRNA level increased by 4- to 5-fold [18]. These findings suggested that the liver-specific changes in lipid metabolism in endotoxemia are at least in part mediated by the regulation of CD36 expression in the liver. These

results also led the authors to hypothesize that, while the FATP-mediated hepatic uptake of FA may be linked to mitochondrial oxidation, CD36 transports FA to the cytoplasm for re-esterification into TG [18]. Conversely, hepatic *SR-B1* mRNA levels showed a prolonged decrease upon LPS administration [15], suggesting distinct roles for CD36 and SR-B1 proteins in hepatocytes, despite both belonging to the same class B family of scavenger receptors [19]. Of note, The SR-B1 protein has been well-studied as a receptor for high-density lipoprotein (HDL) that mediates the selective uptake of HDL-associated cholesterol-ester (CE) [20]. In contrast to SR-B1, CD36 is known to serve as transporter for multiple ligands including FA, oxidized-LDL (ox-LDL), HDL, glycated proteins, serum amyloid A and thrombospondin-1 [21]. Given such differential systemic roles, it seems plausible that they have distinct roles in the liver.

Hepatic CD36 expression has been shown to change quickly by a variety of stimuli, including stress [22] [23]. In acute stress, this change is considered to facilitate lipid uptake of FA derived from plasma TG in combination with the release of lipoprotein lipase into plasma [3] [24]. We found that serum TG levels showed a rapid decrease followed by a rebound at 24 h in our restraint stress model where tail blood sampling was conducted repeatedly on mice fixed in a conical tube. We proposed that this change in TG levels during acute stress may be beneficial in the clearance of bacterial lipids [25]. Notably, some authors have focused on the effect of the acute stress model on the susceptibility to endotoxin shock [26] [27]. However, to our knowledge, the specific implications of such CD36 regulation of stress have not been studied in detail. It is also of clinical interest to study the effect of stress on lipid metabolism not only in healthy animal models/patients but also in animal models/patients suffering from liver dysfunction or inflammation.

We have recently reported that the administration of unmethylated cytidine phosphate guanosine (CpG) dinucleotide, a ligand of Toll-like receptor 9 (TLR9) in combination with D-galactosamine (GalN) can cause relatively liver-specific toxicity and inflammation, compared to the well-studied endotoxemia murine model using LPS where multi-organ injury and systemic inflammation are known to occur [28]. In our CpG/GalN experiments, we employed the same blood sampling scheme that we previously described as causing acute stress that triggers the rapid TG level changes [25]. Therefore, in our CpG/GalN murine model, mice were subjected to two types of stress: restraint stress due to tail blood sampling (denoted as psychological stress although blood sampling-associated physical stresses are also involved) as well as the hepatotoxin-mediated stress that is associated with inflammation.

In this study, we examined the changes in lipid metabolism in our CpG/GalN murine model. Our specific aim was to investigate the presence of synergy in lipid metabolism in the two stress scenarios, namely psychological stress and hepatic inflammation/injury. In the second experiment, we examined changes in *CD36* transcript expression in the CpG/GalN-treated mice (hepatic inflammation/injury

model) in comparison with phosphate-buffered saline (PBS)-injected mice. To gain insight into the differential roles of CD36 and SR-B1, which has been well-studied as an HDL-associated cholesteryl ester (CE) transporter in adrenal glands and liver, we also examined the hepatic gene regulation of *SCARB1*, the gene encoding SR-BI protein, in comparison with *CD36*.

2. Methods

The preparation of mice and details of the experimental procedures were similar to those we have described previously [25] [28]. Briefly, female C57BL/6 mice (7 - 8 weeks old) were given access to food and water *ad libitum* and maintained on a 12-h light/dark cycle (lights on at 8 AM). In the first experiment (the measurement of the lipids parameters) 18 mice were divided into the following three groups (n = 6 for each): namely, "PBS", "CpG" and "CpG/GalN". These groups were intraperitoneally (i.p.) injected with 100 μL PBS, 15 μg CpG in PBS and 15 μg CpG/20 mg GalN in PBS, respectively, as described previously [28]. Blood (50 μL) was sampled from the tail veins of all mice at 1, 5, 8, 24, and 48 h after the i.p. injection. The mice were kept in their cages and, only at the time of blood sampling, were placed inside the restrainer designed in-house from a 50 mL conical polypropylene tube as described previously [25]. Of note, this procedure (*i.e.* the repeated restrain/blood sampling) serves as an acute stressor [25]. We termed the PBS group the psychological stress model, although, strictly speaking, besides the psychological stress, any physiological stress associated with blood loss should also be considered. Serum alanine aminotransferase (ALT), TG and free FA (FFA) levels were measured using Drychem (Fujifilm, Japan) according to the manufacturer's protocol.

In the second experiment (*i.e.* the hepatic transcripts measurement), 42 mice were divided into the three groups of 14 mice, namely, PBS^{liver}, CpG^{liver}, and $CpG/GalN^{liver}$, and each group was further divided into the three groups which were euthanized for the liver sampling at different time points, that is, 8 (n = 4), 24 (n = 4) and 48 h (n = 6) after the i.p. injection performed in the same manner as in the first experiment. Unlike the first experiment, the mice of the second experiment were not subjected to the tail blood sampling and therefore they were largely free from the psychological stress except for the initial i.p. injection until the time for euthanasia. In addition to the three groups, the wild-type mice (n = 5) that were not subjected to any treatment were taken from the cage and euthanized for the liver sampling. Total liver RNA was isolated and measurement of *CD36, SCARB1* and hypoxanthine phosphoribosyl transferase (*HPRT*) transcript copy numbers was performed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously described [25]. Briefly, total liver RNA was purified using the NucleoSpin RNA kit (Macherey-Nagel, Germany). After reverse transcription using random sequence primers, cDNA was subjected to qRT-PCR analysis. The *HPRT* transcript was used as an internal standard. The *CD36*/*HRPT* as well as *SCARB1/HPRT* transcript ratios were measured using TB

Green® Premix Ex Taq™ II (Takara Bio, Japan) following the protocol of the manufacturer. The primer sequences (5′ to 3′) were as follows: *CD36* forward, GGCCAAGCTATTGCGACATG; *CD36* reverse, CCGAACACAGCGTAGATAGAC; *SCARB1* forward, CGTTGTCATGATCCTCATGGT; *SCARB1* reverse, ACAGGCTGCTCGGGTCTAT; *HPRT* forward, TTGTTGTTGGATATGCCCTTGACTA; *HPRT* reverse, AGGCAGATGGCCACAGGACTA. Of note, the *SCARB1* primers were derived from the *SCARB1* nucleotide sequence deposited in GenBank ID: BC004656. 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, Japan.

3. Results

Our recently reported mouse experiments showed that the administration of a combination of CpG/GalN leads to inflammation and tissue injury that is mostly confined to the liver compared with the administration of other TLR ligands [28]. Consistent with this report, mice in the CpG/GalN group showed alanine aminotransferase (ALT) leakage indicating liver injury (**Figure 1(a)**), whereas both the PBS and CpG groups did not show such ALT leakage. This is in agreement with our previous studies showing that GalN sensitizes the effect CpG and therefore this combination causes fulminant hepatitis, while the proinflammatory effect of the CpG injection at this dose alone is not hepatotoxic based on the ALT leakage levels [29]. The administration of 10 μg of CpG in mice has been shown to cause increases in the circulating levels of TNF and IL-6 [30] although these have not been measured in the present study.

In the PBS and CpG/GalN groups, a rapid decrease in the serum TG concentration was observed 1 - 8 h after the initial injection (*i.e.* 0 - 7 h after the first blood sampling) (**Figure 1(b)**). Although the CpG group showed much lower TG levels than the other groups at 1 h likely resulting from a rapid TG decrease immediately after the stress due to the i.p. injection, in the remaining two groups, the serum TG level decreased markedly from ~80 mg/dL to approximately 30 mg/dL at 8 h and exhibited a rebound later (at 48 h). This rapid TG decrease and rebound can be explained as a response to acute psychological stress due to restraint/tail blood sampling as we have reported previously [25]. However, while the TG rebound was approximately 2 - 3-fold of the basal level observed in the PBS group (**Figure 1(b)**), it was less pronounced and slower to occur in the CpG/GalN group, with serum TG levels being greater at 48 h than at 24 h. Strikingly, the CpG group did not display the TG rebound, as shown by the lack of the statistical significance between the 24 and 48 h data, despite the lack of appreciable liver injury based on serum ALT levels (**Figure 1(a)** and **Figure 1(b)**). As our experimental system imposed acute psychological stress during blood sampling [25], we concluded that the changes in serum TG levels triggered by acute stress were abrogated by the CpG treatment. Regarding serum levels of free FAs (FFAs), both CpG and

CpG/GalN groups showed a similar rapid decrease at 8 h similar to the PBS group (Figure 1(c)), but no consistent pattern was observed at 24 and 48 h as the CpG/GalN group showed lower FFA levels while the CpG alone group exhibited higher FFA values compared to the PBS group.

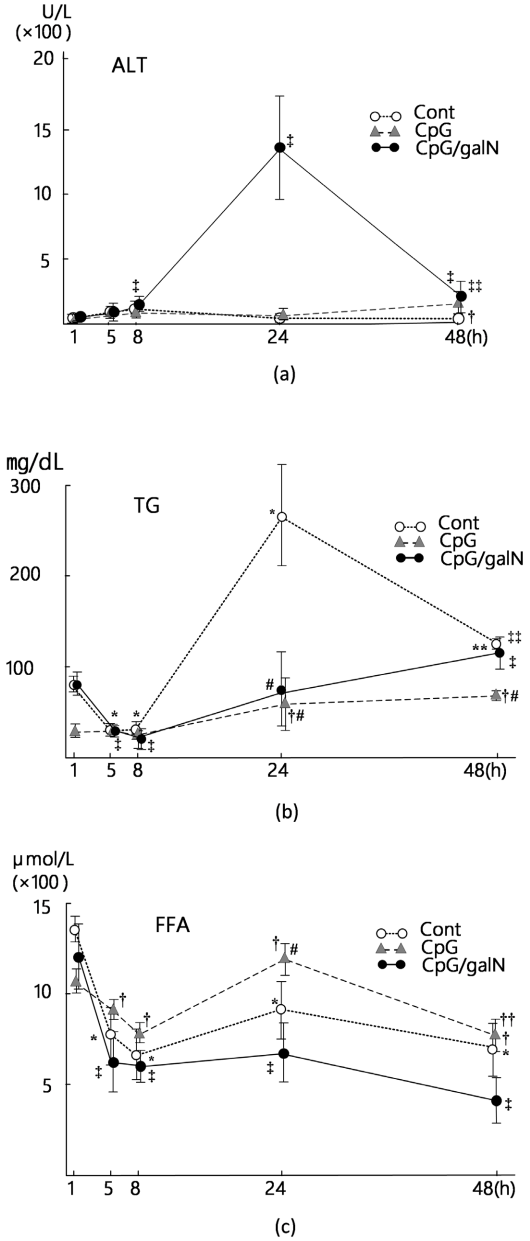

Figure 1. The serum metabolic parameters plotted as a function of the duration after the i.p. injection for the PBS (indicated as "cont"), CpG, and CpG/GalN groups. (a) ALT activity, (b) TG, and (c) free FAs. Notably, the tail blood sampling was performed at 1, 5, 8, 24, and 48 h after i.p. The symbols *, †, and ‡ denote the statistical significance ($p < 0.05$) compared to the corresponding 1 h data for the PBS (control), CpG, and CpG/GalN groups, respectively. The symbols **, ††, and ‡‡ denote the significant difference ($p < 0.05$) between 24 h and 48 h for the PBS (control), CpG, and CpG/GalN groups, respectively. # denotes the difference ($p < 0.05$) between the PBS (Control) and the CpG (and CpG/GalN) group.

Previous studies, including ours, have suggested that CD36 plays an important role in the rapid regulation of serum TG levels [18] [25]. To gain insight into *CD36* gene regulation in the liver-injury model, we measured *CD36* transcript levels in a separately designed experiment. This measurement was carried out in comparison with that of the *SCARB1* transcript that encodes the SR-B1 protein whose expression in the hamster liver has exhibited a prolonged decrease after LPS stimulation [15], even though both SR-B1 and CD36 belong to the class B family of scavenger receptors and that the *CD36* transcript is known to increase after endotoxemia in a mouse model [18].

To gain some insights into the mechanism underlying the changes in the serum TG levels shown above, we measured the *CD36* and *SCARB1* transcript levels in the liver of the mice sacrificed at 8, 24, and 48 h after the i.p. injections of PBS (PBS^{liver} group), CpG (CpG^{liver} group) or CpG/GalN ($CpG/GalN^{liver}$ group). Of note, unlike the first experiment, the mice for this experiment were not subjected to the tail blood sampling and, therefore, they were largely free from the psychological stress until the time for euthanasia, except for the initial i.p. injection.

Figure 2 shows the ratio of (*CD36* transcript level)/(*HPRT* transcript level) after normalizing the ratios using the wild-type mice value as the reference. As the ratios of 5,000 - 15,000 indicate, in both the CpG^{liver} and $C\mathbf{p}G/GalN^{liver}$ groups, a markedly rapid increase in CD36 expression was observed at 8 h after the treatment (**Figure 2(a)**). The *CD36/HPRT* ratio further increased at 48h in both mouse groups, to the points greater compared to the PBS^{liver} mice. Contrary to the *CD36* transcript data, the *SCARB1* transcript did not show such increases. As the small ratios of <0.05 show, their values in the CpG-injected mice (CpG^{liver}) were reduced to very low levels compared to the wild-type mice (**Figure 2(b)**). In the CpG/GalN-injected mice, although some level of expression (~0.2) was observed at 8 h, it decreased at 24 h and 48 h (**Figure 2(b)**). Strikingly, the overall results of the PBS^{liver} were similar to those of $C\mathbf{p}G/GalN^{liver}$ group for both *CD36* and *SCARB1* transcripts. Considering that, in the second experiment, the repeated blood sampling (with the mouse restraint) was not performed, these findings indicate that the initial i.p. injection of PBS alone can cause the stress sufficient for the increase in hepatic *CD36* transcript and the downregulation of the *SCARB1* transcript.

We inspected the individual mice data of the PBS^{liver} group in an attempt to know the cause for the large SD shown in **Figure 2** (**Table 1**). For the PBS^{liver} group, there was a large variance among the mice at 8 h post-injection (**Table 1**), *i.e.* two of the four mice showed increases in *CD36* transcripts while the remaining two mice did not (**Table 1**). A similar variance was seen among the four mice sacrificed at 24 h post-injection, although all of the six mice sacrificed at 48 h post-injection showed elevated CD36 transcript levels. Intriguingly, the pattern of *SCARB1* transcript changes was opposite to that of *CD36*; the increase in *CD36* expression was accompanied by a decrease in the *SCARB1* transcript level. These findings point to an inverse correlation between *CD36* and *SCARB1* expression.

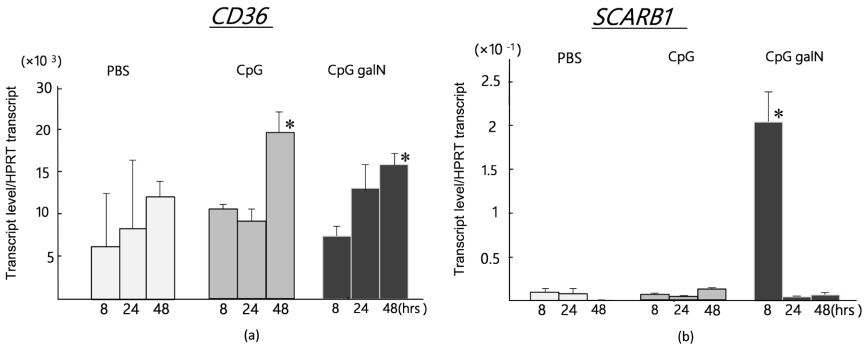

Figure 2. qRT-PCR analyses of *CD36* and *SCARB1* transcripts. The figure shows the ratio of *CD36* transcript/*HPRT* transcript normalized against the value based on five stress-free wild-type mice. The absolute signal intensities for the *HPRT* transcript were on a similar level across all three groups of mice (the range = 23 - 64, the mean = 44.6, and the SD = 12.0), validating the procedures for RNA purification and reverse transcription. For the *CD36* transcript, all 8, 24, and 48 h data showed greater values compared to the wild-type mice data ($p < 0.01$). For the *SCARB1* transcripts, all 8, 24 and 48 h data showed smaller values compared to the wild-type data ($p < 0.01$). For PBS^{liver} mice, the data shown with n.d in **Table 1** were not included and therefore the presented data at 8 and 48 h are not accurate. *shows the significant difference ($p < 0.05$) when compared with the corresponding PBS^{liver} group data, under the assumption that the n.d. data (**Table 1**) can be excluded as an outlier.

Table 1. Hepatic expression of *CD36* and *SCARB1* transcripts for the PBS^{liver} group.

Mouse	Time point	*Normalized CD36/ HPRT*	*Normalized SCARB1/ HPRT*
8-1	8 h	10,919	n.d.*
8-2		12,777	0.003
8-3		2	1.226
8-4		28	1.198
24-1	24 h	13,067	0.001
24-2		18,788	0.010
24-3		0.6	1.655
24-4		0.5	1.427
48-1	48 h	14,792	0.021
48-2		9566	0.003
48-3		10,760	0.002
48-4		13,558	0.003
48-5		10,480	n.d.*
48-6		16,154	0.011

*n.d. represents "not determined" because the PCR product level was lower than the detectable level.

Of note, our finding of a reduction in *SCARB1* mRNA corroborates a previous report where an LPS challenge reduced the hepatic SR-B1 mRNA levels in hamsters [15].

4. Discussion

Accumulating evidence shows that changes in lipid metabolism are involved in the responses to acute psychological stress as well as to stress due to pro-inflammatory challenges in mammals [5] [24]. In real-life scenarios, these two types of stresses, namely, psychological and immunological stresses, can occur simultaneously. In the present study, we used the CpG/GalN murine model to examine the effects of the two types of stresses on circulating lipid parameters. The combination of CpG (a TLR9 ligand) and GalN administration in mice causes acute fulminant hepatitis accompanied by severe hepatic injury and by modest levels of inflammation and injury of non-liver organs [28]. In this model, all three mouse groups were exposed to the restraint stress that is imposed due to our blood sampling procedure. The PBS group exhibited a rapid decrease in the serum TG and FA levels that was followed by a subsequent rebound of serum TG that was caused by mainly psychological stress [25]. Thus, the CpG/GalN mice model represents a realistic scenario where an immunological and psychological stress are combined.

Intriguingly, either a CpG or a CpG/GalN injection was able to abrogate the psychological stress-associated TG rebound observed in the PBS group (**Figure 1(a)**). It is notable that, despite an absence of liver injury (based on ALT release), the CpG group exhibited a severe impairment in the TG rebound compared to the PBS group. Thus, even sub-hepatotoxic levels of CpG injection can cause the abrogation of the psychological stress-associated TG rebound. In the following sections, we discuss the possible mechanisms and the relevance of this finding.

We observed rapid hepatic increases of the *CD36* transcript in the PBS, CpG, and CpG/GalN groups (**Table 1**). This finding is reminiscent of the LPS-induced upregulation of hepatic *CD36* mRNA levels [18]. The increase was more rapid and pronounced in the CpG and CpG/GalN groups compared to the PBS group (**Figure 2** and **Table 1**), supporting the view that *CD36* expression can be upregulated by a variety of stress events and that the degree of *CD36* upregulation is somewhat proportional to the overall stress level: the greater the total stress, the more pronounced the upregulation becomes. An increased CD36 expression appears to be important in the regulation of lipid metabolism in both acute psychological and acute pro-inflammatory stress. In the endotoxemia model mice, higher CD36 levels may facilitate the liver uptake (and disposal) of lipidic toxins (see below for further discussion).

Notably, we observed a reciprocal pattern in the hepatic gene regulation of *CD36* and *SCARB1* (**Table 1**); when the *CD36* expression increased, the *SCARB1* expression decreased, whereas when CD36 expression level remained low, the *SCARB1* expression remained high. Although we have not examined the possible difference between Kupffer cells and hepatocytes in this regard, Kuhovidunkit *et al.* showed that LPS-induced decrease in hepatic SR-B1 mRNA in hamsters is at least in part accounted for by hepatocytes [15]. To our knowledge, the mechanism for the CD36/SR-BI reciprocity has not been studied well. Although our data were

based on a small set of experiments requiring further analysis, it would be relevant to consider the potential benefit of this reciprocity for mammals.

The CD36 and SR-B1 proteins belong to the same class B family of scavenger receptors [19], but their roles are distinct. The SR-B1 protein has been well-studied as a receptor for high-density lipoprotein (HDL) that mediates the selective uptake of HDL-associated cholesterol-ester (CE) [20]. SR-B1 is expressed predominantly in liver and steroidogenic tissues (*i.e.* testis, ovaries, and adrenals) SR-B1 can also bind to other ligands including unmodified low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL), but its role in the cellular uptake of HDL has been best studied [20] [31]. In the liver, SR-B1 is important for the hepatic uptake of CE from HDL, as well as for biliary cholesterol secretion [32]. In contrast to SR-B1, CD36 serves as transporter for multiple ligands including FA, oxidized-LDL (ox-LDL), HDL, glycated proteins, serum amyloid A and thrombospondin-1 [21]. The role of CD36 in FA uptake in heart, skeletal muscles, and adipocytes has been characterized particularly well, but its expression level in liver is negligibly low under normal circumstances [33] [34]. Its role in ox-LDL uptake, formation of foam cells, and contribution to the pro-atherosclerotic state may also be important, but its physiological role in HDL uptake may be less important [35]. Therefore, it seems reasonable to speculate that, while SR-B1 is important in the cellular uptake of HDL-associated CE, CD36 is more important in the uptake of FA (in particular, the FA constituting TG,) in a physiological setting.

In the acute psychological and immunological stresses that work through the innate immune system, it is likely that the physiological roles of CD36 and SR-B1 undergo some changes. In the adrenal gland, the plasma lipoprotein-derived cholesterol (or CE) serves as the precursor for the glucocorticoids crucial for stress response [36], and SR-B1 plays an essential role in the physiological stress-induced increase in adrenal glucocorticoid production [36] [37]. Thus, in our model as well, it is plausible that the stress-induced increase in the adrenal gland production of glucocorticoids requires the SR-B1-mediated uptake of CE from plasma HDL. Given this, the downregulation of SR-B1 expression in the liver upon stress (**Table 1**) may help preserve plasma HDL at high enough levels for the SR-B1-mediated uptake of CE in the adrenal gland that must produce more glucocorticoid hormones.

We hypothesize that hepatic CD36 mediates a rapid uptake of plasma TG-rich lipoproteins or FAs in all three murine groups employed in the present study (**Figure 1(a)**) [25]. We surmise that increased CD36 expression facilitates hepatic uptake of FA derived from circulating TG and the hepatic production and release of TG-rich lipoproteins (e.g. VLDL) into plasma, thereby enhancing the turnover of TG/FA in plasma and facilitating the removal of lipidic toxins that would enter on injury. It may be hypothesized that the CD36-mediated rapid regulation of plasma TG levels is beneficial as it removes lipidic toxins from plasma. We elaborate below on the roles of lipoproteins in neutralization (detoxification) and clearance of bacterial lipidic toxins.

The ability of plasma lipoproteins to inactivate LPS has long been studied. Although this literature comprises few studies, several early reports showed that intravenous administration of reconstituted HDL protects against death in animal models of endotoxic shock (e.g. [38] [39]). Wurfel *et al.* further reported that recombinant-HDL (R-HDL) binds and neutralizes LPS, but that this process was dependent on transfer proteins such as lipid-binding protein (LBP) [40]. At high LBP concentrations, the LBP-mediated transfer of LPS to R-HDL particles has been shown to neutralize LPS [40].

Although these studies drew attention to the HDL-mediated inactivation of LPS, several authors focused on bacterial toxin inactivation by non-HDL-type lipoproteins. For example, Vreugdenhil *et al.* and Flegel *et al.* showed that LDLs are potent for LPS inactivation [16] [41]. The latter authors further showed that chylomicron (CM) and LBP cooperate in reducing LPS toxicity, resulting in decreased cytokine secretion by peripheral blood mononuclear cells in an *in vitro* system [16]. They also showed that LBP induces detoxification of LTA, an immuno-stimulatory component of Gram-positive bacteria, by CM in a dose-dependent manner and that CM exceeded the other lipoproteins in LPS-inactivating capacity in the serum of postprandial humans [16].

Aside from the inactivation (neutralization) of bacterial lipidic toxins by lipoproteins, clearance of LPS (and other lipidic toxins) is another important type of process for prevention of unwarranted inflammation. Notably, Harris *et al.* reported that intravenous administration of *E. coli* endotoxin after preincubation with CM: 1) protects against endotoxin-induced death in rats, 2) increases the clearance rate of endotoxin from plasma and 3) increases the hepatocellular uptake of endotoxin, while shunting endotoxin away from Kupffer cells [17]. Based on these findings, the authors concluded that TG-rich lipoproteins can redirect the metabolism of endotoxins, thereby affording protection against endotoxins. Regarding the molecules mediating LPS clearance, Topchiy *et al.* reported that HepG2 cells clear LPS in a low-density lipoprotein receptor (LDLR)-dependent manner and that primary hepatocytes from Ldlr-/-mice had greatly decreased LPS uptake [42]. Grin *et al.* further showed that LPS and LTA are taken up by the human liver epithelial cell line HepG2 [43]. These studies established the role of LDL and LDLR in LPA and LTA clearance. In the latter study using a proprotein convertase subtilisin/kexin 9-deficient mouse, Grin *et al.* provided data suggesting that LDLR-mediated uptake of bacterial lipids by hepatocytes reduces the availability of bacterial lipid PAMPs to Kupffer cells, thereby reducing cytokine-driven inflammation. Alternatively, it is still possible that lipoproteins may be serving in the clearance of LPS via scavenger receptors [44]. It is notable that, using $Scarb^{I179N}$, a mouse model specifically deficient in hepatic SR-B1, Guo *et al.* showed that hepatic SR-B1 exerts its protection against sepsis through its role in promoting LPS clearance without affecting the inflammatory response in macrophages, demonstrating a critical role for hepatic SR-B1. However, to our knowledge, the potential role of CD36 in clearance largely remains unknown [44].

Given these recent reports and our finding that the TG rebound seen in the PBS group was abrogated by the CpG injection, we hypothesize that the immunological stress (due to pro-inflammatory ligands) can modulate the balance of the uptake and secretion of lipids by the liver in such a way as to shift it to "an uptake-dominant mode". It is possible that, while in the case of psychological stress alone a quick synthesis of the adrenal glucocorticoid hormones may have merit and therefore CD36 expression may rapidly decrease, thereby leading to the TG rebound, that, in turn, may help the preparation for toxin invasion that may follow. Conversely, when the invasion of a significant dose of pro-inflammatory toxins occurs, hepatic CD36 may act to drastically clear the TG-rich lipoprotein, prioritizing the disposal of lipidic toxins. The reduction in hepatic SR-B1 after bacterial toxin challenges may help preserve HDL in the blood that is necessary for glucocorticoid production and regulating the extent of systemic inflammation.

However, despite its clinical relevance, research in this area is still in its infancy. Remaining questions include to what extent hepatic CD36 expressed in the stressed condition expedites the clearance of bacterial lipids. If such hepatic clearance of bacterial lipids through CD36 increases during stress, one may ask whether the uptake by hepatocytes leads to enhanced inflammatory responses from Kupffer cells or whether it rather shunts toxins away from Kupffer cells, thereby reducing the inflammatory response. Although recent attention has been paid to the cooperativity of CD36 with the innate immune system [45], analyses of to what extent the hepatic CD36-mediated toxin clearance can prevent the pro-inflammatory responses will also be warranted.

Although our study focused on the stress, inflammation and lipid metabolism, recent studies have more focused on the signaling pathways triggered by diverse hormones and cytokines, deepening our understanding the lipid metabolism regulation. As an important example, peroxisome proliferator-activated receptors (PPARs) are regulated by FAs and controlling both lipid metabolism and inflammation [46]. A number of studies have also established that the peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α (PGC-1α) is the master regulator of lipid metabolism regulation that promotes hepatic fasting response, including gluconeogenesis, FA beta-oxidation, ketogenesis, and bile-acid homeostasis [47]. The expression of PGC-1α is dynamically regulated in a tissue-specific manner such as cold temperature in brown adipose tissue, fasting in liver or exercise in skeletal muscle [48]. The expression of PGC-1α is regulated in skeletal muscles and the expression in the liver is also important for the metabolism regulation during physical exercise, implicating PGC-1α in the coordination of the systemic metabolism upon physical exercise. This finding has drawn attention to the interest in insulin sensitivity [49]. Relationship between lipid metabolism and inflammation has also been studied mainly with a focus on the transcription factors. For example, FA oxidation is suppressed in sepsis and a decreased level of PPARα expression has been consistently reported in many septic models and humans [50]. Thus, the research frontiers shift to the signaling pathways regulating

lipid metabolism, yet such studies nonetheless help us to discuss the systemic lipid regulation and interplays between organs in many settings.

5. Conclusion

In conclusion, our findings showed that acute stress (caused by the restraint and tail blood sampling) causes a rapid decrease in the serum TG level followed by a rebound in mice, and that this rebound was abolished in the mice with a simultaneous treatment with a TLR 9 ligand (CpG) at a sub-hepatotoxic level. The increase of *CD36* transcript in the liver was induced by the i.p. injection of PBS alone, implying that the stress caused by the i.p. injection can induce hepatic *CD36* expression, but the increase was more pronounced in the mice treated with a subtoxic level of CpG. The hepatic *CD36* transcript increase was accompanied by a decrease in the hepatic *SCARB1* transcript, implying a reciprocal control system operating between the two genes.

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Ethical Statements

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

Conflicts of Interest

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

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List of Symbols

GalN	D-galactosamine
TLR	Toll-like receptor
CpG	unmethylated cytosine-guanosine dinucleotide
ODN	oligodeoxynucleotides
TG	triglycerides
CE	cholesterol ester
SR-B1	scavenger receptor B1