Original Article

Decreased hepatic phosphorylated p38 mitogen-activated protein kinase contributes to attenuation of thioacetamide-induced hepatic necrosis in diet-induced obese mice

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ABSTRACT — We previously reported that thioacetamide (TA)-induced hepatocellular necrosis was attenuated in mice fed a high-fat diet (HFD mice) compared with mice fed a normal rodent diet (ND mice). In this study, we investigated whether p38 mitogen-activated protein kinase (p38 MAPK) was involved in this attenuation. Western blot analysis revealed that hepatic phosphorylated p38 MAPK protein decreased at 8 and 24 hours (hr) after TA dosing in the HFD mice, while it decreased only at 24 hr in the ND mice in comparison to the time- and diet-matched, vehicle-treated mice. p38 MAPK regulates various biological functions including inflammation, therefore, hepatic metabolomics analysis focusing on pro-inflammatory lipid mediators was performed. At 24 hr after TA dosing, only one pro-inflammatory mediator, 12-hydroxyeicosatetraenoic acid (HETE), was higher in the HFD mice. On the other hand, in addition to 12-HETE, 15-HETE and 12-hydroxyeicosapentaenoic acid (HEPE) were higher and omega-3/omega-6 polyunsaturated fatty acids ratios were lower in the ND mice at 24 hr. These results of metabolomics indicated that less pro-inflammatory state was seen in HFD mice than in ND mice at 24 hr. Finally, to confirm whether the observed decrease in phosphorylated p38 MAPK could attenuate TA-induced hepatocellular necrosis, we showed that SB203580 hydrochloride, an inhibitor of p38 MAPK, partially attenuated TA-induced hepatic necrosis in ND mice. Collectively, these results suggest that a prompt decrease in phosphorylation of p38 MAPK after TA administration is one of the factors that attenuate TA-induced hepatic necrosis in HFD mice.

Key words: Mouse, p38 MAPK, High-fat diet, Thioacetamide, Necrosis, Liver

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent disease in developed countries (Cohen *et al.*, 2011). Histopathologically, the livers of NAFLD patients demonstrate steatosis (Cohen *et al.*, 2011), and a similar change is noted in the livers of high fat diet-induced NAFLD model animals (Imajo *et al.*, 2013). We previously demonstrated that thioacetamide (TA), an extensively used hepatotoxicant (Chilakapati *et al.*, 2005), induced hepatic necrosis that was significantly attenuated in mice fed a high-fat diet (HFD mice) compared to mice fed a normal rodent diet (ND mice; Shirai *et al.*, 2013; Shirai *et al.*, 2015). Furthermore, we reported that suppression of TA-induced hepatic oxidative stress by activation of the synthesis of hepatic glutathione was thought

to be one of the factors that attenuate TA-induced hepatic necrosis in HFD mice (Shirai *et al.*, 2015). However, oxidative stress seemed not to be the only cause of TA-induced hepatic necrosis in mice because hepatic necrosis did not completely disappear when the mice were pre-treated with antioxidant before TA dosing (Shirai *et al.*, 2015). This means that factors other than the activated synthesis of hepatic GSH could be involved in the mechanism of the attenuated hepatic necrosis in HFD mice. In addition, compared to ND mice, HFD mice showed more severe hepatocellular swelling at 8 hr after TA dosing and thereafter (Shirai *et al.*, 2013, 2015). In general, swelling is histopathologically considered to represent cellular damage, and we considered that swollen hepatocytes were moderately damaged but were not damaged severely enough for necrosis to occur. Therefore, it was unclear

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why the swelling in HFD mice was so severe at 8 hr while hepatocellular necrosis was slight and comparable to that in the ND mice, and the necrosis in HFD mice was slighter than in ND mice at 24 and 48 hr.

p38 mitogen-activated protein kinase (MAPK) is a kinase that is activated to respond to growth factors or environmental stimuli (Kumar *et al.*, 2003). It was previously reported that p38 MAPK controls cellular volume and inhibition of this enzyme results in cellular swelling (Feranchak *et al.*, 2001; vom Dahl *et al.*, 2001). Furthermore, p38 MAPK is also known to regulate various types of biological events such as cellular differentiation and proliferation, inflammation and apoptosis (Cuenda and Rousseau, 2007; Kumar *et al.*, 2003; Lee *et al.*, 2000). Although the roles of p38 MAPK in the liver have not been completely clarified, some reports indicated that p38 MAPK is involved in hepatotoxicant-induced hepatotoxicity. For example, ethanol administration elicited rapid p38 MAPK activation before hepatic necrosis occurred (Aroor *et al.*, 2010). Likewise, activated p38 MAPK was noted after administration of lipopolysaccharide/D-galactosamine (Zhang *et al.*, 2010) or carbon tetrachloride (Iida *et al.*, 2009). Furthermore, inhibition of p38 MAPK activity reduced liver injury induced by ethanol plus Fas agonistic antibody (Wang *et al.*, 2009), pyrazole plus lipopolysaccharide (Wu and Cederbaum, 2008), or hemorrhagic shock and resuscitation (Lv *et al.*, 2012). These results suggest the possibility that the activity of p38 MAPK changes in HFD animals, and this change induces hepatocellular swelling and modifies hepatic sensitivity of the model animals against TA.

The purpose of this study was to investigate whether

hepatic p38 MAPK contributes to the attenuated TA-induced hepatic necrosis in HFD mice. In this study, the amounts of hepatic total p38 MAPK and phosphorylated p38 MAPK (p-p38 MAPK) protein after TA administration were compared between ND and HFD mice. Hepatic pro-inflammatory lipid mediators were also investigated to examine the influence of the changed level of p-p38 MAPK. In addition, whether a p38 MAPK inhibitor attenuated TA-induced hepatic necrosis was also evaluated in ND mice.

MATERIALS AND METHODS

Chemicals

TA and dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 5-bromo-2-deoxyuridine (BrdU) was purchased from Sigma-Aldrich Co. (Tokyo, Japan), and SB203580 hydrochloride (SB) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Experimental design

In this study, two experiments were performed as described below. The timing of the treatments and the necropsies are outlined in Fig. 1.

Experiment 1 (comparison of hepatic p38 MAPK expression in ND and HFD mice)

Liver samples

Liver samples were collected from animals and some of the results were reported previously (Shirai *et al.*, 2015). Briefly, three-week-old male C57BL/6J mice were

Fig. 1. Schematic diagram of experiments 1 and 2. Each diagram outlines the timing of thioacetamide (TA), 5-bromo-2-deoxyuridine (BrdU) and SB203580 hydrochloride (SB) treatments and necropsies. hr: hours. ND mice: mice fed a normal rodent diet. HFD mice: mice fed a high fat diet.

obtained from Charles River Japan, Inc. (Kanagawa, Japan), and were fed either a normal rodent diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) or a high-fat diet containing 60 kcal% fat (high fat diet 32; Clea Japan, Inc., Tokyo, Japan) and were given tap water *ad libitum*. After 8 weeks of feeding, all mice were given either TA (100 mg/kg body weight; dissolved in saline) or saline once intraperitoneally. The mice were necropsied at 3, 8 and 24 hours (hr) after dosing. In the mice necropsied at 8 and 24 hr, BrdU (100 mg/kg body weight, dissolved with 1% dimethyl sulfoxide/saline) was given intraperitoneally 2 hr before necropsy for additional evaluations (which will be reported separately). After euthanasia, the livers were collected, frozen in liquid nitrogen and stored at -80°C in a deep freezer for the analyses described below.

Western blot analysis of p38 MAPK

For the Western blot analysis of p-p38 MAPK and total p38 MAPK (the sum of p-p38 MAPK and non-phosphorylated p38 MAPK), liver cytosol was prepared using nuclear and cytoplasmic extract reagents (NE-PER; Thermo Fisher Scientific Inc., Rockford, IL, USA). The protein concentration of the cytosol was adjusted to 10 mg/mL with phosphate-buffered saline and subsequently diluted to 5 mg/mL with Tris-SDS beta-mercaptoethanol sample loading buffer (Cosmo Bio Co., Ltd., Tokyo, Japan). Then samples were heated at 95°C for 5 min, and 10 µL (50 µg) of each sample was loaded onto 10% polyacrylamide gel (Funakoshi Corporation, Tokyo, Japan) and subjected to electrophoresis. The proteins were transferred from the gel to an Immobilon® polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA) using a blotting apparatus (Horizeblot; Atto Corporation, Tokyo, Japan). This membrane was blocked with enhanced chemiluminescence (ECL) blocking agent (GE Healthcare, Buckinghamshire, UK) and treated successively with primary antibodies. Anti-human p-p38 MAPK (Thy 180/Tyr 182) and p38 MAPK antibodies (Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies. Then, the membrane was treated with biotin-labeled anti-rabbit IgG antibody (GE Healthcare) as the secondary antibody and finally treated with streptavidin-horseradish peroxidase conjugate (GE Healthcare). Protein-antibody complexes were detected using ECL Western blotting detection reagent (GE Healthcare) and the membrane was exposed to instant film (Fujifilm Corporation, Tokyo, Japan). The protein bands were scanned with AE-6961 (Atto Corporation, Tokyo, Japan) and the intensity was quantified with densitometry analyzer (CS

Analyzer, Atto Corporation). The results of the quantification were expressed as percentage of the value of the vehicle-treated ND mice at each time point.

Metabolomics analysis of pro-inflammatory lipid mediators in the liver

To investigate inflammatory state in the liver, a metabolomics analysis was performed at Metabolon Inc. (Durham, NC, USA). The results regarding glutathione metabolism was reported previously (Shirai *et al.*, 2015). In brief, the samples from the frozen liver were prepared using an automated MicroLab STAR® system (Hamilton Company, Salt Lake City, UT, USA). The samples were then separated into three equal aliquots for analysis in three independent platforms: ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Metabolites were identified by automated comparison of the ion features in the experimental samples with a reference library of purified standards; these features included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as their associated MS/MS spectra. Among the identified metabolites, metabolites relating to pro-inflammatory lipid mediators were investigated.

Experiment 2 (the effect of p38 MAPK inhibitor SB on TA-induced hepatotoxicity in ND mice)

In-Life experiments and histopathological examination

Eight-week-old male C57BL/6J mice were obtained from Charles River Japan, Inc., and were fed a normal rodent diet. At 11 weeks of age, all of the mice were given either TA (100 mg/kg) or saline once intraperitoneally as described in *Experiment 1*. To inhibit hepatic p38 MAPK function, the mice were given SB (75 mg/kg body weight; dissolved with saline) or saline twice intraperitoneally, once at 1.5 hr before and again at 6 hr after TA or saline administration in reference to the previous reports, in which *in vivo* p38 MAPK inhibition was performed by SB (Tibbles *et al.*, 2002; Cao *et al.*, 2004; Badger *et al.*, 1996). Hence, there were 4 groups, vehicle-only treated group (vehicle/vehicle group), SB- and vehicle-treated group (SB/vehicle group), vehicle- and TA-treated group (vehicle/TA group) and SB- and TA-treated group (SB/TA group). All of the mice were necropsied at 24 hr after administration, and the left lateral lobe and right and left medial lobes of the liver were trimmed, fixed in 10% neutral buffered formalin and embedded in paraffin for histopathology.

Histopathological examination

Histopathological specimens were prepared and stained with hematoxylin and eosin (HE). As reported previously (Shirai *et al.*, 2013, 2015), TA-induced centrilobular necrosis of hepatocytes and centrilobular swelling of hepatocytes were graded as described below.

Centrilobular necrosis of hepatocytes

- Grade 1: Centrilobular necrosis that reached zone 2 was observed in less than 5 lobules in the 3 lobes (the left lateral, right medial and left medial lobes).
- Grade 2: Centrilobular necrosis that reached zone 2 was observed in 5 or more lobules, or centrilobular necrosis that reached zone 1 was observed in less than 5 lobules in the 3 lobes.
- Grade 3: Centrilobular necrosis that reached zone 1 was observed in 5 or more lobules in the 3 lobes, and the necrotic area was less than 33% of 1 or more of the 3 lobes.
- Grade 4: The necrotic area was 33% to 66% of 1 or more the 3 lobes.
- Grade 5: The necrotic area was more than 66% of 1 or more of the 3 lobes.

Centrilobular swelling of hepatocytes

- Grade 1: Centrilobular swelling was observed, but it was limited to the centrilobular area in the hepatic lobe (zone 3).
- Grade 2: Centrilobular swelling that reached zone 2 was observed in less than 50% of lobules.
- Grade 3: Centrilobular swelling that reached zone 2 was observed in 50% or more of lobules.

Ethics

All of the studies were approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd., and were conducted in compliance with the "Law Concerning the Protection and Control of Animals" (Japanese Law 105, October 1, 1973; revised on June 22, 2005).

Statistical analysis

The results are expressed as the mean $\pm$ S.D. In analysis of the Western blot data, the F-t test was performed. The homogeneity of variance was evaluated by the F test, and the Student's t test (for homogeneous data) or the Aspin-Welch t test (for heterogeneous data) was performed using Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA). In analysis of histopathological data, the Mann-Whitney U test was performed using the common gateway interface program (<http://aoki2.si.gunma-u.ac.jp/exact/utest/getpar.html>). In analysis of

metabolomics data, a log transformation was applied to the observed relative concentrations for each metabolite because, in general, the variance increases as a function of the average response of the metabolite. After log transformation, a three-way ANOVA was performed using Array Studio (Omicsoft Corp., Cary, NC, USA) and/or "R" (<http://cran.r-project.org/>), and multiple comparisons were performed with the false discovery rate (FDR) method. Each FDR was estimated using the q-values. Statistical analysis was not performed on the ratios of arachidonate/eicosapentaenoate and linoleate/eicosapentaenoate. We used the conservative criteria of $p < 0.05$ and $q < 0.05$ to judge statistical significance in all statistical analyses.

RESULTS

P-p38 MAPK and total p38 MAPK protein expression levels in HFD and ND mice (Experiment 1)

We previously reported that, severe hepatocellular necrosis was noted in the ND mice at 24 and 48 hr, but hepatocellular necrosis was significantly attenuated in the HFD mice (Shirai *et al.*, 2015). To examine whether p38 MAPK was involved in this attenuation in the HFD mice, the amounts of total p38 MAPK and p-p38 MAPK protein were measured by Western blot analysis. In the TA-dosed HFD mice, p-p38 MAPK almost disappeared at 8 hr after dosing (Fig. 2). At 24 hr, p-p38 MAPK apparently diminished in both the TA-dosed ND mice and HFD mice. Densitometry analysis revealed that the amount of p-p38 MAPK in the TA-administered HFD mice was significantly lower than that of the vehicle-treated HFD mice or that of the TA-treated ND mice at 8 hr (Fig. 3A). There

Fig. 2. Western blots of hepatic total p38 MAPK and phospho-p38 MAPK in the livers of the vehicle- and TA-treated mice. The number of mice fed the high-fat diet (HFD) or normal rodent diet (ND) was 3 at each observation time. hr: hours. p-p38 MAPK: phospho-p38 MAPK.

Decreased p-p38 MAPK attenuated liver necrosis in obese mice

Fig. 3. The relative amounts of phospho p38 MAPK (A) and total p38 MAPK 8 (B) are shown in Fig. 2. The amounts of these proteins were calculated by densitometry analysis and the amounts were expressed as percentages of the value of the vehicle-treated ND mice at each time point. The number of mice fed the high-fat diet (HFD) or normal rodent diet (ND) was 3 at each observation time. White columns: vehicle-treated ND mice. White columns with black shading: TA-dosed ND mice. Red columns: vehicle-treated HFD mice. Red columns with black shading: TA-dosed HFD mice. *, **: $p < 0.05$ and $p < 0.01$ for comparison between the vehicle-treated ND mice and the TA-dosed ND mice, or between the vehicle-treated HFD mice and the TA-dosed HFD mice (student's t-test). †: $p < 0.05$ for comparison between the vehicle-treated ND mice and the TA-dosed ND mice, or between the vehicle-treated HFD mice and the TA-dosed HFD mice (Aspin-Welch t-test). ‡: $p < 0.05$ for comparison between the TA-dosed ND mice and the TA-dosed HFD mice (Aspin-Welch t-test).

was no significant difference between TA-dosed ND mice and vehicle-treated ND mice at this time point. The p-p38 MAPK blots of the TA-dosed ND and HFD mice were not detected at 24 hr (Fig. 3A). As for total p38 MAPK, at 3 hr, significantly lower expression in the TA-dosed ND mice and significantly increased expression in the TA-dosed HFD mice were observed compared with the vehicle-treated ND mice and vehicle-treated HFD mice, respectively (Fig. 3B). At each time point, there were no statistical differences between the vehicle-treated ND and HFD mice for the amounts of either p-p38 MAPK or total p38 MAPK (Fig. 3A and 3B).

Metabolomics analysis of pro-inflammatory lipid mediators in the liver (Experiment 1)

Table 1 shows the results of the metabolomics analysis on unsaturated fatty acids and their metabolites, which have been reported to relate to pro-inflammation state (Tam, 2013; Cunningham *et al.*, 1985; Calder, 2009; Pilkington *et al.*, 2014; Strassburg *et al.*, 2012; Elabdeen *et al.*, 2013; Kalogeropoulos *et al.*, 2010). These mediators were measured at 8 and 24 hr after TA administration.

At 8 hr after TA dosing, 12-HETE was significantly higher in the HFD mice compared to the vehicle-treated

HFD mice. At 24 hr, 12-HETE was significantly higher in both the HFD and ND mice compared with the vehicle-treated ND mice and vehicle-treated HFD mice, respectively. In addition, 15-HETE and 12-HEPE were higher and eicosapentaenoate, linoleate and arachidonate were significantly lower in the ND mice. Furthermore, the ratios of eicosapentaenoate/linoleate and eicosapentaenoate/arachidonate were apparently lower only in the ND mice, while they were higher or the same in the HFD mice at 24 hr.

The effect of treatment with p38 MAPK inhibitor SB on TA-induced hepatic necrosis in ND mice (Experiment 2)

To investigate whether p38 MAPK attenuates TA-induced hepatic necrosis in mice, a selective p38 MAPK inhibitor, SB, was given to the ND mice either by itself or together with TA. With SB treatment, none of the ND mice died by 24 hr after TA dosing, while 3 out of 9 ND mice given TA without SB died (Table 2). Moreover, although TA induced centrilobular hepatocellular necrosis with or without SB treatment, SB significantly attenuated the severity of TA-induced hepatic necrosis at 24 hr (Figs. 4A and 4B, Table 2).

Table 1. Hepatic pro-inflammatory lipid mediators.

Biochemical Name	KEGG	HMDB	8 hr				24 hr			
			ND		HFD		ND		HFD	
			vehicle	TA	vehicle	TA	vehicle	TA	vehicle	TA
Eicosapentaenoate (20:5n3)			4.4733	4.479	0.5043	0.3287	7.1776	1.1081*	0.5694	0.6777
Linoleate (18:2n6)			0.8808	1.0202	0.9648	0.8172	1.2018	0.6707**	1.0004	1.197
Arachidonate (20:4n6)			0.8244	0.9639	2.0286	1.5333	0.7994	0.4497**	1.9545	1.7596
5-HETE			1.3093	1.0107	0.5728	0.5633	1.5248	2.7212	0.8697	0.9554
12-HETE		HMDB06111	0.9185	1.3386	0.4028	0.9371**	0.8294	6.7603**	0.5735	3.8228**
15-HETE	C04742	HMDB02110	0.9736	0.9003	0.7659	0.6818	1.1193	2.7601*	0.8332	1.2954
12-HEPE		HMDB10202	1.3675	1.5867	0.6441	0.7395	1.6396	5.2847**	0.6714	0.8476
Eicosapentaenoate/linoleate ratio			5.08	4.39	0.52	0.4	5.97	1.65	0.57	0.57
Eicosapentaenoate/arachidonate ratio			5.43	4.65	0.25	0.21	8.98	2.46	0.29	0.39

8 hr and 24 hr: Animals necropsied at 8 or 24 hr after vehicle or thioacetamide (TA) administration, respectively (n = 5). ND: mice fed a normal rodent diet, HFD: mice fed a high fat diet. KEGG: Kyoto Encyclopedia of Genes and Genomes, HMDB: Human Metabolome Database. Each value was re-scaled to have median equal to 1. Statistical analysis was not performed on eicosapentaenoate/linoleate and eicosapentaenoate/arachidonate ratios. *, **: p < 0.05 or p < 0.01 in the value of each metabolite versus time-matched vehicle-treated group (three-way ANOVA).

Table 2. The effect of SB203580 hydrochloride administration on thioacetamide-induced hepatic lesions at 24 hr after dosing in mice fed a normal rodent diet.

Hours after TA administration	vehicle/vehicle	SB/vehicle	vehicle/TA	SB/TA
Mice Fed a Normal Rodent Diet				
Number of mice ^a	6 (0)	6 (0)	9 (3)	9 (0)
Incidence of necrosis ^b	6, 0, 0, 0, 0, 0	6, 0, 0, 0, 0, 0	0, 0, 0, 0, 6, 3	0, 0, 2, 5, 1, 1
Mean score	0.0 ± 0.00	0.0 ± 0.00	4.3 ± 0.50	3.1 ± 0.93**
Incidence of swelling ^b	6, 0, 0, 0	6, 0, 0, 0	9, 0, 0, 0	9, 0, 0, 0
Mean score	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

vehicle/vehicle group: group treated only with vehicle, SB/vehicle group: group treated with SB203580 hydrochloride (SB) and vehicle, vehicle/TA group: group treated with vehicle and thioacetamide (TA), SB/TA group: group treated with SB and TA. Mean score is the mean of grades for individual mice ± S.D.. a: The values in parentheses show the number of dead mice. b: The numbers show the incidence of each grade (0 to 5 for hepatocellular necrosis and 0 to 3 for hepatocellular swelling), with the leftmost number representing grade 0. **: p < 0.01 for comparison versus vehicle/TA group (Mann-Whitney U test).

Fig. 4. Histopathological appearance of typical hepatic lesions at 24 hours after thioacetamide (TA) administration in a mouse fed a normal rodent diet. A: The liver of a mouse given TA and vehicle. B: The liver of a mouse given TA and SB203580 hydrochloride. CV: central vein, PV: periportal vein; HE stain; Bar = 100 micrometer.

DISCUSSION

We previously reported that severe hepatocellular necrosis was noted in ND mice at 24 and 48 hr, but hepatocellular necrosis was significantly attenuated in HFD mice (Shirai *et al.*, 2013, 2015). On the other hand, hepatocellular swelling was noted both in ND and HFD mice, but the severity of the swelling was significantly higher in HFD mice at 8 to 48 hr. In this study, we demonstrated that p-p38 MAPK, which is the phosphorylated active form of p38 MAPK (Kumar *et al.*, 2003), was significantly lower in the livers of the HFD mice at 8 and 24 hr after TA administration in comparison to the vehicle-treated HFD mice at the same time point. In the ND mice, such decrease was observed only at 24 hr. p38 MAPK is a multifunctional protein kinase and its biological role is both beneficial and harmful (Cuenda and Rousseau, 2007). The function of p38 MAPK in the liver has not been fully unveiled, but it was reported that p38 MAPK seemed to be involved in hepatotoxicity induced by some hepatotoxicants (Aroor *et al.*, 2010; Zhang *et al.*, 2010; Iida *et al.*, 2009). In addition, inhibition of p38 MAPK is known to attenuate hepatic injury (Lv *et al.*, 2012; Wang *et al.*, 2009; Wu and Cederbaum, 2008). These results suggested the possibility that decreased p38 MAPK activity promptly after TA administration could contribute to the attenuation of TA-induced hepatic necrosis in HFD mice.

p38 MAPK plays an important role in inflammation, and treatment with a p38 MAPK inhibitor decreases the inflammatory response (Kumar *et al.*, 2003; Lee *et al.*, 2000). To investigate whether inflammatory activity was reduced together with decreased phosphorylation of p38 MAPK protein in HFD mice after TA administration, lipid mediators related to pro-inflammation were investigated by metabolomics analysis. Compared to the vehicle-treated HFD mice, at 24 hr after TA administration, only one pro-inflammatory mediator, 12-HETE (Cunningham *et al.*, 1985), was higher in the HFD mice. On the other hand, in addition to 12-HETE, 2 pro-inflammation-related metabolites, 15-HETE (Calder, 2009) and 12-HEPE (Pilkington *et al.*, 2014; Strassburg *et al.*, 2012), were higher in the ND mice in comparison to the vehicle-treated ND mice. Moreover, the omega-3 polyunsaturated fatty acid eicosapentaenoate and the omega-6 polyunsaturated fatty acids, linoleate and arachidonate (Chen *et al.*, 1996), were significantly lower and the ratio of omega-3/omega-6 polyunsaturated fatty acids was apparently lower only in the ND mice at 24 hr. It was previously reported that a decrease in the ratio of omega-3/omega-6 polyunsaturated fatty acids may be a marker of pro-inflammation because omega-3 and omega-6 polyunsaturated fat-

ty acids induce and reduce inflammation, respectively (Elabdeen *et al.*, 2013; Kalogeropoulos *et al.*, 2010). Collectively, our results demonstrated that livers of HFD mice exhibited less pro-inflammatory state than those of ND mice at 24 hr after TA administration, and this result was consistent with the decrease in phosphorylated p38 MAPK observed only in the TA-dosed HFD mice at 8 hr.

Although our results indicated that a decrease in p38 MAPK activity attenuated TA-induced hepatic necrosis in HFD mice, there have been no investigations of the contribution of p38 MAPK activity to TA-induced hepatic necrosis in the mouse. Hence, to investigate the contribution of p38 MAPK to TA-induced hepatic necrosis in the mouse, the selective p38 MAPK inhibitor SB was administered to ND mice before TA dosing. In this study, SB treatment reduced mortality, and attenuated TA-induced hepatic necrosis in the ND mice at 24 hr. Our results showed that hepatic p38 MAPK activity was one of the causes of TA-induced hepatic necrosis in mice, and therefore, promptly decreased phosphorylation of p38 MAPK protein after TA administration was thought to contribute to the attenuation of TA-induced hepatic necrosis in HFD mice.

In the previous study, we demonstrated that the HFD mice exhibited more severe hepatocellular swelling after TA administration at 8 hr (Shirai *et al.*, 2013, 2015). Swelling is induced by osmotic imbalance and is histopathologically considered to be a toxic change. However, our previous study demonstrated that, in comparison to the ND mice, hepatocellular necrosis was slighter in the HFD mice at 24 hr or thereafter (Shirai *et al.*, 2015). In addition, one kind of biosyntheses, glutathione synthesis, was more active in the livers of the HFD mice at 8 hr. These results indicated that this severe swelling observed in the HFD mice at 8 hr was not a simple and temporal severe toxic change, but was a change occurring by not only TA toxicity but also some additional biological reactions. It was reported that p38 MAPK inhibition delayed and diminished volume regulatory K⁺ efflux and induced swelling of hepatocyte under hypoosmotic exposure (vom Dahl *et al.*, 2001). Furthermore, p38 MAPK inhibition increased Na⁺ currents through cell membranes and enlarged cell volume in HTC rat hepatoma cell line (Feranchak *et al.*, 2001). p38 MAPK inhibition resulted in cellular swelling, therefore, there was a possibility that TA-induced hepatocellular swelling in HFD mice at 8 hr was caused, at least in part, by inhibition of p38 MAPK activity. Severe hepatocellular swelling was also noted in the HFD mice at 24 hr and the hepatic p38 MAPK was not phosphorylated at this time point. However, the involvement of p38 MAPK at this time point seemed to

be limited because hepatic p-p38 MAPK disappeared but hepatocellular swelling was slight in the ND mice.

As for the other changes, at 3 hr after TA dosing, total p38 MAPK significantly decreased in the ND mice and significantly increased in the HFD mice, compared with the vehicle-treated ND and HFD mice, respectively. The cause of this increase or decrease is unknown, but these changes seemed to have little biological meaning because the active form of p38 MAPK, p-p38 MAPK, did not change either in the ND or HFD mice at this time point. In metabolomics analysis, the pro-inflammatory mediator 12-HETE significantly increased at 8 hr only in the HFD mice. However, this increase was much slighter than the increase of 12-HETE in the ND and HFD mice at 24 hr. Furthermore, the other pro-inflammatory lipid mediators or the ratio of omega-3/omega-6 polyunsaturated fatty acids did not change in the HFD mice at 8 hr. Therefore, the increase of 12-HETE noted in the TA-dosed HFD mice at 8 hr was not considered to indicate an activated pro-inflammatory state compared with the TA-dosed HFD mice at 24 hr or the TA dosed ND mice at 8 and 24 hr.

In this study, there were no differences in the hepatic p-p38 MAPK between the vehicle-treated ND and HFD mice at 3, 8 and 24 hr. Hence, the phosphorylation of p38 MAPK seemed not to be affected in the HFD mice. In turn, the response against TA administration differed between these types of mice. Our study indicates the possibility that the susceptibility to hepatotoxicants is modified in NAFLD due to an alteration in the status of phosphorylation of p38 MAPK following hepatotoxicant exposure. This is the first study that addresses decreased p38 MAPK activity in NAFLD animals after TA dosing.

In conclusion, it is suggested that a prompt decrease in phosphorylated p38 MAPK after TA administration is one of the factors that attenuate TA-induced hepatocellular necrosis in HFD mice.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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