Diabetic KK-AY mice are highly susceptible to oxidative hepatocellular damage induced by acetaminophen

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Submitted 4 September 2009; accepted in final form 3 June 2010

Kon K, Ikejima K, Okumura K, Arai K, Aoyama T, Watanabe S. Diabetic KK-AY mice are highly susceptible to oxidative hepatocellular damage induced by acetaminophen. Am J Physiol Gastrointest Liver Physiol 299: G329–G337, 2010. First published June 10, 2010; doi:10.1152/ajpgi.00361.2009.—Despite pathophysiological similarities to alcoholic liver disease, susceptibility to acetaminophen hepatotoxicity in metabolic syndrome-related nonalcoholic steatohepatitis (NASH) has not been well elucidated. In this study, therefore, we investigated acetaminophen-induced liver injury in KK-AY mice, an animal model of metabolic syndrome. Twelve-week-old male KK-AY and C57Bl/6 mice were injected intraperitoneally with 300 or 600 mg/kg acetaminophen, and euthanized 6 h later. Liver histology was assessed, and hepatic expression of 4-hydroxy-2-nonenal was detected by immunohistochemistry. Levels of reduced glutathione were determined spectrophotometrically. Phosphorylation of c-Jun NH2-terminal kinase (JNK) was analyzed by Western blotting. Hepatocytes were isolated from both strains by collagenase perfusion, and cell death and oxidative stress were measured fluorometrically by use of propidium iodide and 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester, respectively. Acetaminophen induced more severe necrosis and apoptosis of hepatocytes in KK-AY mice than in C57Bl/6 mice and significantly increased serum alanine aminotransferase levels in KK-AY mice. Acetaminophen-induction of 4-hydroxy-2-nonenal in the liver was potentiated, whereas the levels of reduced glutathione in liver were lower in KK-AY mice. Acetaminophen-induced phosphorylation of JNK in the liver was also enhanced in KK-AY mice. Exposure to 20 μM tert-butyl hydroperoxide did not kill hepatocytes isolated from C57Bl/6 mice but induced cell death and higher oxidative stress in hepatocytes from KK-AY mice. These results demonstrated that acetaminophen toxicity is increased in diabetic KK-AY mice mainly due to enhanced oxidative stress in hepatocytes, suggesting that metabolic syndrome-related steatohepatitis is an exacerbating factor for acetaminophen-induced liver injury.

Drug-induced liver injury; metabolic syndrome; steatohepatitis; oxidative stress; hepatotoxicity

ACETAMINOPHEN OVERDOSE is the leading cause of acute liver failure in the United States (22). Acetaminophen toxicity accounts for ~50% of all cases of acute liver failure in the United States and carries a 30% mortality (35). In the development of acetaminophen hepatotoxicity, overdosed acetaminophen is metabolized by cytochrome P450 (CYP) 2E1 (25) and forms a chemically reactive metabolite, N-acetyl-p-benzoquinonimine (NAPQI). NAPQI reacts with glutathione (GSH) (20) thereby forming an acetaminophen-GSH conjugate (6) and GSH depletion (29). Acetaminophen has long been recognized as a dose-dependent toxin, and most cases of acetaminophen-induced liver failure are suicidal overdose more than 15 g; however, in some cases, people develop acute liver failure although they do not take doses of acetaminophen exceeding the amount recommended on the package labeling of up to 4 g daily (22). Since unintentional acetaminophen-induced liver injury tends to be treated late with antidote N-acetylcysteine, it is extremely important to investigate the risk factor for increasing susceptibility to acetaminophen-induced liver injury.

Nonalcoholic steatohepatitis (NASH) is the syndrome diagnosed following liver biopsy results that are consistent with alcoholic hepatitis and/or fibrosis although patients deny significant alcohol use. NASH has been consistently associated with metabolic syndrome including obesity, diabetes mellitus, hypertension, dyslipidemia, and insulin resistance (18, 26, 38, 42), and it has been proposed that simple steatosis can progress to NASH (7). Recently, it was reported that nonalcoholic fatty liver disease (NAFLD) conveys a nearly fourfold increase of risk for liver injury caused by various drugs. Some exacerbating drug treatments include antihypertensive medication, drugs that inhibit platelet aggregation, antimicrobials, nonsteroidal anti-inflammatories, and proton pump inhibitors prescribed to obese middle-aged patients (41). Although it is well known that alcohol use is an important risk factor of acetaminophen-induced liver injury (33, 43), the impact of NAFLD on acetaminophen hepatotoxicity still remains unclear.

KK-AY mice are a strain derived from crossing the diabetic KK mouse (13) with the lethal yellow (A′) mouse, which carry a mutation of the agouti (a) gene in chromosome 2 (28, 40). KK-AY mice exhibit phenotypes including obesity, dyslipidemia, and insulin resistance, which resemble metabolic syndrome in human (1, 11, 15). We have reported that KK-AY mice develop steatohepatitis spontaneously and exhibit increased susceptibility to methionine- and choline-deficiency diet-induced steatohepatitis (32). In the present study, we investigated the sensitivity of KK-AY mice to acetaminophen hepatotoxicity to address the influence of steatosis and steatohepatitis on acetaminophen-induced liver injury.

MATERIALS AND METHODS

Animals and experimental design. Male KK-AY and C57Bl/6 mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in air-conditioned, specific pathogen-free animal quarters with lighting from 0800 to 2100 and were given unrestricted access to a standard laboratory chow and water throughout this study. All animals received humane care, and the experimental protocol was approved by the Committee of Laboratory Animals according to institutional guidelines. C57Bl/6 mice, which are the strains of two generations ago, were selected as nonobese and nondiabetic controls. After acclimation, both KK-AY and C57Bl/6 mice at 12 wk of age were separated into three groups randomly, and some mice were injected with 300 or...
600 mg/kg acetaminophen intraperitoneally. Control groups were injected with physiological saline solution alone. After administration of acetaminophen for 6 h, mice were killed by exsanguination by snipping the inferior vena cava, followed by collection of liver and serum samples.

**Histological analysis.** For histological evaluations, liver tissues were fixed in 10% buffered formalin and embedded in paraffin, and hematoxylin-eosin staining was performed. To detect apoptotic cell death in tissue, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed by using a commercial kit according to the manufacturer’s instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche, Indianapolis, IN). TUNEL-positive staining was assessed by using a green nuclear fluorescence dye and was compared with a total nuclei stain, propidium iodide (PI). Staining was quantified by use of laser scanning confocal microscopy (Zeiss 410; Carl Zeiss, Thornwood, NY) and was performed on more than 500 hepatocytes per animal. TUNEL staining was expressed as the number of positively stained nuclei divided by the total number of nuclei.

**Immunohistochemistry.** The expression and localization of tissue 4-hydroxy-2-nonenal (4-HNE) in the liver was detected by immunohistochemical staining as previously described elsewhere (32). Briefly, deparaffinized tissue sections were incubated with a monoclonal anti-4-HNE antibody (Japan Institute for the Control of Aging, Nikken SEIL, Shizuoka, Japan) and a secondary biotinylated antimouse IgG. The specific binding was visualized with the avidin-biotin complex solution followed by incubation with a 3,3-diaminobenzidine tetrahydrochloride solution by use of Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Specimens for histology and immunohistochemistry were observed under an optical microscope (PH-2; Olympus, Tokyo, Japan) equipped with a digital microscope camera (VB6000; Keyence, Osaka, Japan).

**Measurement of serum aminotransferase levels.** Serum alanine aminotransferase (ALT) levels were measured spectrophotometrically.
by a standard enzymatic method using a commercial kit (KAINOS Laboratories, Tokyo, Japan).

Lipid peroxidation assay and measurement of GSH in the liver. The tissue contents of malondialdehyde (MDA)/4-hydroxyalkenals (HAE) were measured colorimetrically using the lipid peroxidation assay kit (Calbiochem, EMD Biosciences, San Diego, CA). Briefly, the whole liver was homogenized in ice-cold phosphate-buffered saline (PBS) containing 5 mM butylated hydroxytoluene and was centrifuged at 3,000 g for 10 min to collect the supernatant. Samples were then incubated with N-methyl-2-phenylindole in methanol:acetonitrile and methanesulfonic acid at 45°C for 60 min, and the absorbance at 586 nm was measured spectrophotometrically. Reduced GSH levels in the liver tissue samples were measured using a commercial kit (OXIS International, Portland, OR) according to the manufacturer’s instructions.

Western blot analysis. Protein extracts were obtained by homogenizing frozen tissues in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, and protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany) followed by centrifugation at 17,400 g for 15 min, and the protein concentration was determined by use of a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Five micrograms of protein was separated in 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated with a primary rabbit polyclonal anti-phospho-stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) (Thr183/Tyr185) antibody (Cell Signaling Technology, Danvers, MA), followed by a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (DakoCytomation Norden). Subsequently, specific bands were visualized using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were stripped by incubation in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, at 50°C for 30 min and reprobed with rabbit anti-SAPK/JNK polyclonal antibody (Cell Signaling Technology) and secondary HRP-conjugated anti-rabbit IgG antibody.

Isolation and primary culture of mouse hepatocytes. Hepatocytes were isolated from both strains by in situ collagenase perfusion and differential centrifugations as previously described (21). Hepatocytes were resuspended in Waymouth’s medium MB-752/1 containing 2 mM l-glutamine, 10% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell viability was greater than 90%, as determined by Trypan blue exclusion. Hepatocytes were plated in a 24-well microtiter plates (1.5 × 10^5 cells per well) or 35-mm Petri dishes (6 × 10^5 cells per dish, Falcon, Lincoln Park, NJ). Plates and coverslips were coated with 0.1% Type 1 rat-tail collagen. Hepatocytes were preincubated in humidified 5% CO2-95% air at 37°C, and medium was replaced with Krebs-Ringer-HEPES buffer (KRH) containing 115 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM KH2PO4, 1.2 mM MgSO4, and 25 mM HEPES (pH 7.4) at 37°C after overnight incubation (19).

Fluorometric assay of cell viability and oxidative stress in primary cultured hepatocytes. Cell death and production of oxidative stress in isolated hepatocytes was determined fluorometrically by using PI and 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydrofluorescein diacetate (CMH2DCF), respectively. After attachment to 24-well plates, hepatocytes were washed once and replaced with KRH buffer containing 30 μM PI. Fluorescence was measured by using a multwell fluorescence reader (Fluoroskan Ascent, Thermo Fisher Scientific, Waltham, MA), as previously described elsewhere (31). Cell death assessed by PI fluorometry correlates closely with Trypan blue exclusion and enzyme release as indicators of oncotic necrosis.

Oil red O staining. Triglycerides in hepatocytes were visualized by Oil Red O staining. Overnight cultured hepatocytes were fixed
with 4% formaldehyde for 10 min and then stained with Oil Red O for 1 h followed by washing with 60% methanol and PBS. Cells were photographed by using a phase-contrast microscope equipped with a digital sight camera system (DS-5M-L1, Nikon, Japan).

Statistical analysis. Morphometrical and densitometric analyses were performed with Scion Image (version Beta 4.0.2, Scion, Fredrick, MD). Data were expressed as means ± SE. Statistical differences between means were determined by Student’s t-test, one-way analysis of variance (ANOVA), or Kruskal-Wallis ANOVA on ranks followed by an all-pairwise multiple-comparison procedure (Student-Newman-Keuls method) as appropriate. P < 0.05 was selected before the study to reflect significance.

RESULTS

Acetaminophen causes severe liver injury in KK-A^y mice. At first, we evaluated the sensitivity to acetaminophen-induced hepatotoxicity in KK-A^y mice by injecting two different doses (300 or 600 mg/kg) of this drug intraperitoneally. Mild liver steatosis was observed in 12-wk-old KK-A^y mice without administration of acetaminophen as expected (Fig. 1B). A single injection of the lower dose of acetaminophen (300 mg/kg), which did not affect liver histology in C57Bl/6 mice (Fig. 1C), caused mild necrotic liver injury with infiltration of inflammatory cells predominantly in pericentral area at 6 h

![Effect of acetaminophen on apoptotic cells death in the liver in KK-A^y mice.](image)

Fig. 3. Effect of acetaminophen on apoptotic cells death in the liver in KK-A^y mice. Apoptotic cells in the liver 6 h after a single injection of 600 mg/kg acetaminophen were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Representative photomicrographs of the liver from C57Bl/6 mice saline controls (A), KK-A^y mice saline controls (B), C57Bl/6 mice treated with acetaminophen (C), and KK-A^y mice treated with acetaminophen (D) are shown (original magnification ×100). Number of TUNEL-positive hepatocytes was counted, and average percentages of TUNEL-positive cells from 5 different animals are plotted. More than 500 cells per 1 animal were counted (E). *P <0.05 vs. C57Bl/6+acetaminophen by ANOVA on ranks and Student-Newman-Keuls post hoc test.
A higher dose of acetaminophen (600 mg/kg), which induced mild liver injury even in C57Bl/6 mice (Fig. 1E), caused extremely severe necrosis in the liver in KK-Ay mice (Fig. 1F).

Serum ALT levels were not changed in C57Bl/6 mice 6 h after treatment with 300 mg/kg acetaminophen (24 ± 1 IU/l) and increased significantly to 47 ± 4 IU/l in KK-Ay mice (Fig. 2A, left, P < 0.05). A higher dose of acetaminophen (600 mg/kg) elevated serum ALT levels to 3,281 ± 513 IU/l in C57Bl/6 mice, whereas the levels were increased significantly to 8,707 ± 1,400 IU/l in KK-Ay mice (Fig. 2B, left, P < 0.05) as expected. Furthermore, the ratio of acetaminophen-induced increases in ALT levels vs. control values in each strain was plotted (Fig. 2, A and B, right). Elevations in serum ALT levels were potentiated significantly in KK-Ay mice by both lower and higher doses of acetaminophen, indicating that KK-Ay mice are more susceptible to acetaminophen-induced liver injury.

To determine whether apoptotic cell death is involved in hepatocyte injury caused by acetaminophen, TUNEL staining was performed (Fig. 3). A few TUNEL-positive cells were observed in the liver in C57Bl/6 mice 6 h after treatment with 600 mg/kg acetaminophen (Fig. 3C), where the percentage of TUNEL-positive hepatocytes were 5.7 ± 0.7% (Fig. 3E). In KK-Ay mice after acetaminophen, the percentage of TUNEL-positive cells and necrotic cell death increased in the area

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**Fig. 4.** Effect of acetaminophen on lipid peroxidation and glutathione content in the liver in KK-Ay mice. Hepatic expression of 4-HNE was detected by immunohistochemistry. Representative photomicrographs from C57Bl/6 mice (A) and KK-Ay mice (B) treated with 6 h after a single injection of 600 mg/kg acetaminophen are shown. Malondialdehyde (MDA)/4-hydroxyalkenals (4-HAE) levels in liver homogenates were measured colorimetrically (C); n = 5, *P < 0.05 by ANOVA on ranks and Student-Newman-Keuls post hoc test. Glutathione (GSH) levels in liver homogenates were measured colorimetrically (D); n = 5, *P < 0.05 by ANOVA on ranks and Student-Newman-Keuls post hoc test. Values are normalized by total protein concentrations in the homogenates.
around central vein (Fig. 3D) and the percentage of TUNEL-positive cells was increased significantly to 16.9 ± 2.4% (P < 0.05).

Oxidative stress following acetaminophen treatment is potentiated in KK-A^Y mice. Next, we evaluated lipid peroxidation in liver tissue of both strains after treatment with acetaminophen. Immunohistological staining revealed that 600 mg/kg acetaminophen induced a much higher amount of 4-HNE in liver tissue of KK-A^Y mice compared with C57Bl/6 mice (Fig. 4B). The localization of 4-HNE expression denoted the same tendency of localization as the area of necrotic and apoptotic cell death. To further evaluate oxidative stress in the liver quantitatively, the amount of MDA plus HAE was measured colorimetrically. Interestingly, the amount of MDA plus HAE in the liver from KK-A^Y mice was significantly higher than C57Bl/6 mice consistent with higher basal levels prior to acetaminophen treatment in KK-A^Y mice. Although hepatic expression of MDA plus HAE was increased in both strains after treatment with acetaminophen, it was further enhanced in KK-A^Y mice as expected (Fig. 4C). These findings indicated that the levels of lipid peroxide following acetaminophen treatment were higher in KK-A^Y mice than in C57Bl/6 mice.

Interestingly, the hepatic content of reduced GSH, one of the most important physiological radical scavenger, before and 6 h after administration of acetaminophen was measured. Reduced GSH was decreased significantly in the liver of KK-A^Y mice compared with C57Bl/6 mice in basal levels before administration of acetaminophen (P < 0.05). Reduced GSH in the liver was dramatically decreased after treatment with acetaminophen in both strains (Fig. 4D).

Acetaminophen-induced activation of JNK is enhanced in KK-A^Y mice. Since previous studies indicated that activation of JNK is involved in acetaminophen-induced liver injury (10, 39), we measured phosphorylation of JNK in liver tissue after treatment with acetaminophen by Western blotting. Although acetaminophen treatment increased significantly expression of hepatic phospho-JNK, expression was enhanced more than twice in KK-A^Y mice compared with C57Bl/6 mice (Fig. 5).

Hepatocytes from KK-A^Y mice are more susceptible to oxidative stress-mediated cell death. To evaluate the direct effect of acetaminophen on hepatocytes, hepatocytes were isolated from liver of both strains, cultured, and exposed to 10 mM acetaminophen. The viability of isolated hepatocytes was not different between both strains and was more than 87%. In hepatocytes isolated from C57Bl/6 mice, basal levels of dichlorofluorescein diacetate (DCF) fluorescence after 5 h were 624 ± 19% over the initial levels, whereas DCF fluorescence was increased to 1,017 ± 104% in the presence of 10 mM acetaminophen after 5 h as expected. In contrast, cells from KK-A^Y mice, which contained obvious lipid droplets in cytoplasm (Fig. 6A), demonstrated higher basal increment (1,135 ± 21%) as well as enhanced elevation in DCF fluorescence to Fig. 5. Effect of acetaminophen on activation of JNK in the liver in KK-A^Y mice. Expression of JNK and phospho-JNK (p-JNK) in hepatic tissue 6 h after injection of 600 mg/kg acetaminophen was detected by Western blotting. Representative photographs of 46 kDa and 54 kDa bands for p-JNK and JNK are shown (A). The ratio of densitometrical values of specific bands for p-JNK to JNK are plotted (B); n = 5, *P < 0.05 vs. C57Bl/6+acetaminophen by ANOVA on ranks and Student-Newman-Keuls post hoc test.

Fig. 6. Effect of acetaminophen on oxidative stress in primary-cultured hepatocytes from KK-A^Y mice. Viability of hepatocytes isolated from both strains was confirmed by Trypan blue staining (A) (original magnification ×100). Overnight-cultured hepatocytes were incubated in Krebs-Ringer-HEPES buffer supplemented with 10 μM CMH2DCFDA for 30 min, and then exposed to 10 mM acetaminophen. Oxidative stress was evaluated by the increment of dichlorofluorescein diacetate (DCF) fluorescence. Values represent the average of total cell fluorescence expressed as the percentage of basal fluorescence before addition of acetaminophen (B); n = 4, *P < 0.05 vs. C57Bl/6 control, #P < 0.05 vs. C57Bl/6+acetaminophen by ANOVA on ranks and Student-Newman-Keuls post hoc test.
1,974 ± 19% 5 h after addition of acetaminophen (Fig. 6B).

Acetaminophen induced oxidative stress in hepatocytes from KK-A′ mice in dose-dependent manner whereas acetaminophen did not increase oxidative stress to hepatocytes isolated from KK-A′ mice as young as 8 wk old (Fig. 7B). The treatment with a JNK inhibitor transiently prevented acetaminophen-induced oxidative stress in hepatocytes from KK-A′ mice in earlier time points up to 4 h but finally lost inhibitory effects at 5 h (Fig. 7C).

Exposure to 20 μM tert-butyl hydroperoxide (t-BuOOH), which induces NAD(P)H oxidation and mitochondrial reactive oxygen species (ROS) generation (4), led to cell death in 52% of primary cultured hepatocytes from KK-A′ mice whereas it did not cause any cell death in cells from C57Bl/6 mice (Fig. 8A). Exposure to t-BuOOH (20 μM) for 30 min induced a 1.4 times higher oxidative stress response in hepatocytes from KK-A′ mice compared with cells from C57Bl/6 mice (Fig. 8B). These findings indicated that hepatocytes from KK-A′ mice are more susceptible to t-BuOOH-induced oxidative stress and subsequent necrotic cell death than cells from C57Bl/6 mice.

**DISCUSSION**

KK-A′ mice spontaneously develop steatohepatitis due to insulin resistance, which resembles the pathophysiological features of human NASH (32). Our data in the present study demonstrated that KK-A′ mice are more susceptible to acetaminophen-induced liver injury involving both necrosis and apoptosis of hepatocytes (Figs. 1–3) compared with C57Bl/6 mice. These findings clearly indicated that steatohepatitis associated with insulin resistance increases the susceptibility to acetaminophen hepatotoxicity.

In acetaminophen-induced liver injury, the sequence of events leading to injury in hepatocytes is based on metabolism of acetaminophen, which precedes an inflammatory response involving the activation of the innate immune system and subsequent regenerative response of the liver (3, 10, 23). Oxidative stress caused by the reactive metabolite NAPQI is a key element in the early phase of acetaminophen-induced liver injury (16, 17). In the present study, after treatment with acetaminophen lipid peroxides content in the liver was higher in KK-A′ mice than in C57Bl/6 mice (Fig. 4, A–C), indicating that increased oxidative stress caused severe necrosis in the liver of KK-A′ mice. Recently,
increasing attention has been suggested for the role of phosphorylation of JNK in the pathogenesis of acetaminophen-induced liver injury (10). JNK in hepatocytes is thought to be activated by oxidative stress and stimulates the cell death pathway (5, 12). It has been reported that a JNK inhibitor blocks acetaminophen-induced Bax translocation from cytosol to mitochondria and prevents death of mouse hepatocytes (10). In the present study, phosphorylation of JNK in the liver was more enhanced in KK-Ay mice than in C57Bl/6 mice (Fig. 5). Next, we examined the direct effect of acetaminophen to isolated hepatocytes from both strains. As expected, acetaminophen induced greater oxidative stress in primary-cultured hepatocytes from KK-Ay mice, which shows a close similarity to results in vivo (Fig. 6B). Collectively, the results demonstrated that enhanced oxidative stress followed by phosphorylation of JNK in hepatocytes causes exacerbation of acetaminophen-induced liver injury in KK-Ay mice. Acetaminophen did not induce oxidative stress in hepatocytes with few lipid droplets from younger (8 wk old) KK-Ay mice (Fig. 7, A and B). This finding suggests that fatty accumulation in hepatocytes contributes to the increment of oxidative stress in hepatocytes in KK-Ay mice. Furthermore, a JNK inhibitor transiently prevented acetaminophen-induced oxidative stress in hepatocytes from KK-Ay mice (Fig. 7C), supporting the hypothesis that JNK plays a role in acetaminophen-induced oxidative stress in the early, regulated phase, whereas the latter phase of enhanced oxidative stress is independent of JNK.

Interestingly, basal expression levels of hepatic MDA plus HAE were higher in KK-Ay mice compared with C57Bl/6 mice (Fig. 6D), which reflects increased ROS generation in hepatocytes (37). Taken together, these data indicate that constitutive ROS generation in the untreated state is potentiated in steatotic hepatocytes in KK-Ay mice. Importantly, KK-Ay mice showed a significantly lower content of reduced GSH in the liver. This lower level in the untreated state sets the stage for susceptibility for injury since it is well known that GSH is one of the most potent natural ROS scavengers (21) (Fig. 4D). Thus the mechanisms underlying basal increases in hepatic ROS in KK-Ay mice most likely involve both enhanced production of ROS and impairment of ROS scavenger system. These findings are consistent with recent studies showing that ROS generation is increased in the liver of NASH patients (34, 36). It has also been reported that the livers of NASH patients contain less GSH compared with healthy persons (24) or have an alteration in GSH metabolism (2). These findings indicate that increased generation of ROS on a routine basis and decreased GSH content contribute to the enhanced oxidative stress in the liver of KK-Ay mice.

Although acetaminophen is oxidized by CYP2E1 in particular and is changed to a reactive metabolite, we previously confirmed that the basal expression levels of CYP2E1 mRNA are not different between C57Bl/6 and KK-Ay mice (32). Therefore, we hypothesized that hepatocytes of KK-Ay mice are more sensitive to oxidative stimuli. To test this hypothesis, we evaluated the effect of t-BuOOH, an oxidant chemical, on hepatocytes isolated from KK-Ay mice. In general, t-BuOOH at concentrations over 100 μM induce NAD(P)H oxidation, increase mitochondrial Ca2+ uptake, and elicit mitochondrial ROS production (4, 30), which subsequently cause the mitochondrial permeability transition, thus leading to cell death in isolated rat hepatocytes (14). In the present study, we used concentrations as low as 20 μM t-BuOOH to induce minimal generation of mitochondrial ROS in hepatocytes isolated from C57Bl/6 mice. In contrast, the same amount of t-BuOOH induced severe oxidative stress and necrotic cell death in hepatocytes from KK-Ay mice (Fig. 8). These findings indicate that hepatocytes from KK-Ay mice produce more oxidative stress by ROS-inducing stimulation (i.e., t-BuOOH) compared with those from C57Bl/6 mice. Recently, increasing lines of evidence have indicated that oxidative stress is involved in progression from simple steatosis to NASH (8, 9, 27). Therefore, it is hypothesized that enhanced sensitivity to ROS-inducing chemicals such as acetaminophen in steatotic hepatocytes participates in the pathophysiology of NASH as one of the second hits (7).

In conclusion, acetaminophen causes enhanced oxidative stress and phosphorylation of JNK, thereby inducing more severe liver injury in diabetic KK-Ay mice. Steatotic hepatocytes in these mice appear to be more susceptible to ROS-generating stimuli including acetaminophen and t-BuOOH, in part through significant decreases in reduced GSH. Our experimental data suggest that metabolic syndrome-related hepatic steatosis is a potential risk factor for acetaminophen-induced liver injury.

ACKNOWLEDGMENT

The authors thank Professor Ko Okumura MD, PhD, and colleagues (Department of Immunology, Juntendo University School of Medicine) for collaboration on in vitro analyses.

GRANTS

This work was supported in part by Grant-in-Aid (Nos. 18790474 and 20790508 to K. Kon; 16590633, 15909791, and 21590859 to K. Ikejima; and 18390213 and 21390234 to S. Watanabe); and High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare of Japan (to S. Watanabe); and grants from Liver Forum in Kyoto (cosponsored by Viral Hepatitis Research Foundation in Japan and Dainippon Sumitomo Pharma, to K. Ikejima), Takeda Science Foundation (to K. Ikejima), and Research Conference on Alcohol and Health (sponsored by Suntory, to K. Ikejima).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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