role in induction of steatosis, inflammation, and fibrosis in the liver. More recent experimental and clinical data suggest that stimulation of the innate immune system plays a key role in the development and progression of ALD.[2–5] Gut-derived endotoxin, a component of the gram-negative bacterial wall, has been proposed as a key player in the pathogenesis of ALD. Acute ethanol administration reduced activation of Kupffer cells by gut-derived endotoxin, which plays a pivotal role in alcoholic liver injury.[6–8] For example, alcoholics with ALD have high serum endotoxin levels, and serum endotoxin levels correlate with ALD severity.[6]

In recent years, a significant role for proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), in the onset of liver disease has been indicated both by clinical observations of an enhanced circulating level of TNF-α and other cytokines in patients with ALD, and by results of studies with animal models of alcohol-induced liver damage.[9,10] On the other hand, immune host-bacteria interactions have also begun to be better characterized. It was shown that innate immune cells recognize conserved molecular patterns associated with pathogens through patterns recognized with receptors, among which the family of Toll-like receptors (TLR) occupy an important place.[11] TLR combine with the pattern recognition molecule CD14 to form a complex (TLR-4-CD14) that activates the NF-κB pathway; thus, soluble CD14 (sCD14) levels are strongly associated with endotoxin levels. Endotoxin induces production of TNF-α by Kupffer cells *via* TLR-4 and contributes to liver injury.

Non-alcoholic fatty liver disease (NAFLD) is a condition with a wide spectrum of liver damage, including steatosis, which can have a good prognosis despite its potential to progress to steatohepatitis, with or without fibrosis and cirrhosis.[12,13] NAFLD is a major chronic liver disease in adults and children, affecting 20–30% of the general population in Western countries. The condition is strongly associated with insulin resistance (IR), obesity, metabolic syndrome, and type 2 diabetes mellitus. Non-alcoholic steatohepatitis (NASH)[14,15] is an important liver disease because it is an intermediary stage in the development of fatty liver disease which can progress to cirrhosis and hepatocellular carcinoma. Its pathological course resembles that of alcohol-induced liver injury, but the condition occurs in patients who do not abuse alcohol. The histological characteristics of NASH resemble those of alcoholic steatohepatitis (ASH), suggesting that the two diseases have a similar pathogenesis. NASH is associated with metabolic syndrome, *i.e.*, obesity, diabetes, dyslipidemia, and IR. According to a review by Starley *et al.*,[16] the exact mechanism behind the development of hepatocellular carcinoma (HCC) in cases of NASH remains unclear, although the pathophysiologic mechanisms behind the development of NASH related to IR and the subsequent inflammatory cascade likely contribute to the carcinogenic potential of NASH (**Fig. 1**). It is now believed that NASH can progress to fibrosis, cirrhosis, and eventually HCC. Although reports of HCC complicating NASH have been accumulating, its precise characteristics are still controversial. NASH may account for a large proportion of cases of idiopathic or cryptogenic cirrhosis, which predisposes patients to the development of HCC. An interesting working model,[13] known as the "two-hit" theory, postulates a progression from simple steatosis to NASH, fibrosis, or cirrhosis. The "first hit" consists of the accumulation of excessive hepatic fat owing to IR. This often occurs in patients with metabolic syndrome, and although not sufficient to cause NASH, it is enough to predispose the liver to chronic inflammation. The oxidative stress owing to ROS, gut-derived endotoxin, and soluble mediator synthesized both from cells of the immune system and from cells of the adipose tissue have been indicated as risk factors responsible for the "second hit."

A recognized effect of ethanol is the ability to enhance oxidative stress. Ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH) in the cytosol, by cytochrome P450 2E1 (CYP2E1) in the endoplasmic reticulum, and by catalase in the peroxisomes.[17] In addition, the ADH-mediated metabolism of ethanol generates a reduced form of nicotinamide adenine dinucleotide (NADH), which promotes steatosis by stimulating the synthesis of fatty acids and opposing their oxidation. Rodent models of ALD are all characterized by significantly increased expression of the major alcohol-metabolizing enzyme CYP2E1, and it has been suggested that the oxidative metabolism of ethanol by CYP2E1 contributes significantly to the development of ALD through the generation of free radicals/ROS.[13,17,18] The most common cause of CYP2E1 expression is early alcoholic liver damage, such as alcoholic steatosis and alcoholic steatohepatitis. CYP2E1, however, is also induced to express in NASH, which can be understood on the basis of the physiologic role of CYP2E1.[17,19]

Our previous review showed a common pathogenic mechanism in the development of non-alcoholic as well as alcoholic steatohepatitis.[20] The mechanism is that increased endotoxin levels cause the activation of Kupffer cells through TLR-4, leading to increased expression of TNF-α and increased levels of ROS. These events induce inflammation and fibrosis, progressing to NASH. The elevation in the hepatic tissue and plasma levels of TNF-α in cases of NASH has been attributed to a combination of secretion by fat cells associated with obesity and secretion by Kupffer cells activated by endotoxin. In combination with data from clinical studies, these findings indicate that TNF-α mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver damage. Although there have been several excellent reviews of the role of the innate immune system in ALD and NAFLD/NASH,[2,5,7,9,13,20–25] in this review, in contrast, we put forward the concept of a role for gut-derived bacterial

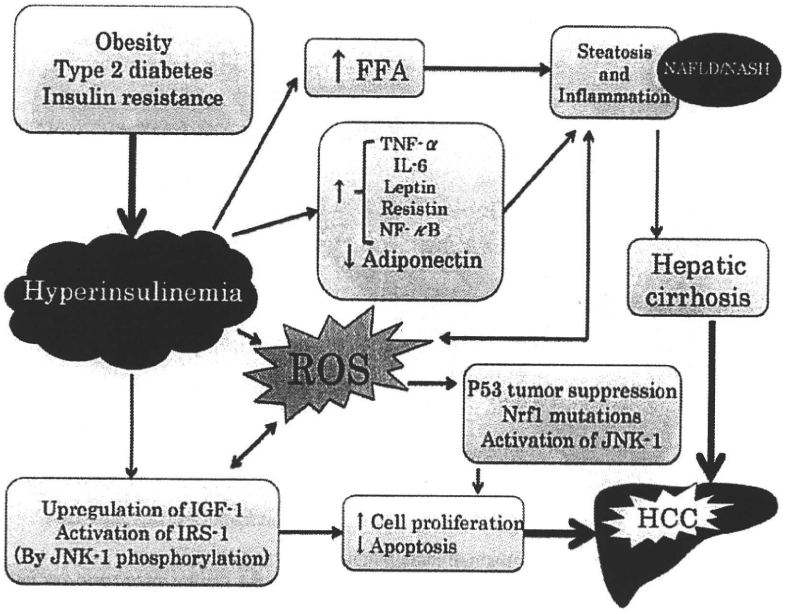

Fig. 1. Development of HCC from NASH
Insulin resistance and the subsequent inflammatory cascade associated with the development of NASH appear to play a significant role in the carcinogenesis of HCC. The occurrence of complications of NASH, including cirrhosis and HCC, is expected to increase with the growing epidemic of diabetes and obesity. On the other hand, the development of NASH is also associated with oxidative stress and the release of reactive oxygen species (ROS), which likely contribute to the development of HCC. FFA, free fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; IGF-1, insulin-like growth factor-1; IRS-1, insulin receptor substrate-1; Nrf1, nuclear respiratory factor-1; JNK-1, c-Jun N-terminal kinase 1.

toxins, the innate immune system, and oxidative stress in the chronic liver disease caused by a common pathogenic mechanism in ALD and NAFLD/NASH.

Bacterial Translocation and ALD

The role of endotoxin in ALD has been shown in many studies.[2,5–10,25] Bacterial translocation (BT) from the gastrointestinal tract, *i.e.*, spillover endotoxemia, is important to the relationship between endotoxin and hepatotoxicity, since the clearance of endotoxin may be due to rapid uptake in the reticuloendothelial system (RES), especially by Kupffer cells in the liver. Endotoxin derived from Gram-negative bacteria in the intestinal microflora normally penetrate the mucosa only in trace amounts, enter the portal circulation, and become cleared from the liver to maintain the control of immune homeostasis. Resident macrophages (Kupffer cells) and hepatocytes both contribute to this process through different endotoxin recognition systems. There is a positive correlation between liver dysfunction and the occurrence of BT, and the clearance of endotoxin from the circulation is decreased in a state of hepatic dysfunction. Rush *et al.*[26] and Deith *et al.*[27] reported increased permeability of the gut under shock conditions, with the spread of endotoxin or bacteria into the bloodstream and translocation into other organs, and emphasized the role of gut barrier failure. BT induced by hemorrhagic shock is an etiologic factor in the pathogenesis of multiple organ failure. In a recent study, Mori *et al.*[28] suggested that the lipid peroxidation of intestinal neutrophils is involved in BT during hemorrhagic shock and that a free radical scavenger, edaravone, is potentially useful in diminishing BT after hemorrhagic shock. It is therefore possible that alcohol-induced hepatocellular damage occurs as a result of bacterial or endotoxin translocation facilitated by a reduction of RES function in ALD. The progression of ALD is a complex phenomenon, as it not only results from the direct effects of alcohol and its metabolites, but involves other factors such as a leaky gut, which results in endotoxemia.[25] Gut-derived bacterial endotoxin has now been implicated as an important cofactor in the progression of alcohol-induced liver injury.

Roles of Kupffer Cells in ALD

Gut-derived endotoxin and depression of RES function: Most administered endotoxin is located in cells of the RES in animals, particularly in Kupffer cells and splenic macrophages. The ability of Kupffer cells to eliminate and detoxify various exogenous and endogenous substances (*e.g.*, endotoxin) is an important physiological regulatory function. Hepatic uptake and detoxification is important for preventing systemic reactions to gut-derived endotoxin. It has been proposed that endotoxins are taken up initially by Kupffer cells and then by hepatocytes. Several

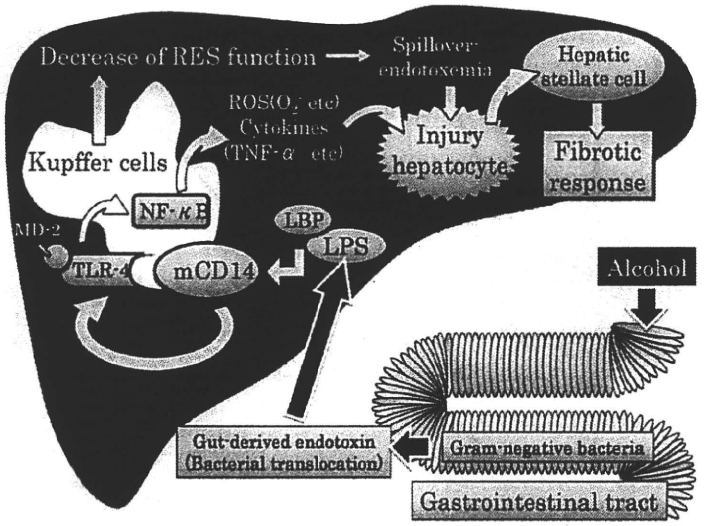

Fig. 2. Role of Kupffer cells and gut-derived endotoxin in alcoholic liver injury
It has been shown that acute ethanol administration reduces activation of Kupffer cells. On the other hand, activation of Kupffer cells by gut-derived endotoxin plays a pivotal role in alcoholic liver injury. Following chronic alcohol ingestion, endotoxin released from certain intestinal bacteria moves from the gut into the bloodstream and into the liver. Endotoxin, also called lipopolysaccharide (LPS), in association with soluble LPS-binding protein (LBP), interacts with a receptor complex consisting of the mCD14 (membrane-associated CD14) and Toll-like receptor-4 (TLR-4). This interaction causes the production of the regulatory nuclear factor kappa B (NF-κB), which in turn leads to the generation of significant amounts of cytotoxic factors, namely free radicals (O_2- *etc.*) and various cytokines, most prominently TNF-α. TNF-α has been shown to be an essential factor in the injury to primary liver cells (*i.e.*, hepatocytes) associated with alcoholic liver disease (ALD).

observations support the hypothesis that Kupffer cells are involved in hepatic injury caused by ethanol. Chronic alcohol abuse impairs the phagocytic activity of the hepatic RES and depresses the clearance of gut-derived endotoxin by Kupffer cells.[25,29] Several authors have suggested that gut bacteria play a crucial role in the development of ALD.[25] Not only the inactivation of RES, which reduces the clearance of endotoxin, but also an increase in the absorption of endotoxin from the intestine may be involved in mechanisms of ethanol-induced endotoxemia. The consumption of a large amount of alcohol increases the risk of hemorrhagic erosion in the stomach both in experimental animals and in humans.[29]

Several mechanisms may underlie the significant increase in endotoxin levels in the bloodstream following chronic alcohol use. In addition, increased absorption of endotoxin from the intestine plays a role in ALD. Alcohol disrupts the gastrointestinal barrier function and subsequently induces the diffusion of luminal bacterial products into the portal blood. The damaging effect of alcohol on the mucosa of the upper gastrointestinal tract is associated with an enhanced permeability of the gut mucosa. On the other hand, direct evidence of increased translocation of endotoxin across the gut mucosa caused by ethanol was obtained in experiments using rats.[30] A pronounced increase in the translocation of endotoxin from the gut lumen into the portal blood caused by alcohol was demonstrated. Acute ethanol ingestion, especially at high concentrations, facilitated the absorption of endotoxin from the rat small intestine *via* an increase in intestinal permeability, which may play an important role in the endotoxemia observed in alcoholic liver injury.[31] When ethanol-fed rats received endotoxin by constant infusion into a peripheral vein, ALD did not potentiate, despite markedly increased plasma endotoxin levels, suggesting the development of tolerance to endotoxin in these animals.[32] In patients with ALD, plasma endotoxin levels are high compared with those in normal subjects and patients with non-alcoholic cirrhosis.[33]

Activation of Kupffer cells by gut-derived endotoxin: As noted above, gut-derived endotoxin plays an important role in alcoholic liver injury. Attention has been directed toward the effect of ethanol ingestion on Kupffer cell function, which is stimulated by gut-derived endotoxin *via* mechanisms dependent on increased gut permeability, and the possible relationship between Kupffer cells and alcohol-induced liver injury. In an intragastric model of chronic ethanol administration, the development of liver injury progressed with an increase in TNF-α, associated with an increase in serum endotoxin levels. As reviewed by Wheeler,[7] when activated, Kupffer cells produce signaling molecules (*i.e.*, cytokines) that promote inflammatory reactions as well as ROS, which can damage liver cells (**Fig. 2**). Interestingly, oxidative stress induced by alcohol also causes modifications of hepatic proteins, which trigger immune reactions. Previous studies have shown that the

activation of Kupffer cells is involved in alcohol-induced liver injury, that plasma endotoxin levels increased in rats on the Tsukamoto-French protocol, and that levels correlated well with the pathology.[34,35] The activation of Kupffer cells results from exposure to endotoxin derived from the cell wall material of Gram-negative bacteria in the gut. An elevated circulating endotoxin level has been detected in heavy drinkers as well as in experimental animals after chronic ethanol administration. Following the ingestion of ethanol, significant alterations occur in RES function, which results in increased host susceptibility to infection. Kupffer cells comprise the largest resident macrophage population within the RES, and this tissue-fixed macrophage population is a key component of the inflammatory response and produces many mediators that initiate, perpetuate, and modulate this response.

The macrophages, when stimulated with endotoxin, release numerous cytokines. The increased release of pro-inflammatory mediators (*e.g.*, TNF-α, interleukin (IL)-1, IL-6, and ROS) and infiltration of other inflammatory cells (*e.g.*, neutrophils) eventually cause liver damage. Among inflammatory cytokines, TNF-α is a critical factor in ALD, and the hypothesis has been confirmed in animal models and human studies.[2,7,33] It appears that early alcoholic liver injury involves interactions of cytokine over-production due to induction of a "hyper-inflammatory" state in monocytes/macrophages and sensitization of hepatocytes to cell death. The activation of Kupffer cells is a prominent event in the initiation of ALD. Patients with alcoholic hepatitis (AH) have elevated serum TNF-α levels, and the elevated serum TNF-α levels have been correlated with a poor prognosis in AH. This assumption was supported by the finding that antibodies against TNF-α attenuate alcohol-induced liver injury in rats. Direct evidence for a central role of TNF-α in the pathogenesis of ALD stems from experiments using TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) knockout mice. Thurmans' laboratory[36] suggested that long-term ethanol feeding caused liver injury in wild-type and TNF-R2 knockout mice but not in TNF-R1 knockout mice, providing solid evidence in support of the hypothesis that TNF-α plays an important role in the development of early alcohol-induced liver injury *via* the TNF-R1 pathway. On the other hand, accumulating evidence suggests that an imbalance between the activities of pro-inflammatory and anti-inflammatory mediators contributes to ALD. Among the pro-inflammatory mediators, TNF-α plays a critical role in the pathogenesis of ALD; TNF-R1 knockout mice are resistant to the toxic effects of alcohol exposure.[37] It is apparent that in alcoholic liver injury, the Kupffer cells respond to stimulation by gut-derived endotoxin and apoptotic cells in the tissue resulting in an increased inflammatory response. Therefore, it was concluded that these results strongly support the hypothesis that increased TNF-α release from activated Kupffer cells plays an important role in the development of ALD.[20]

Signaling Pathways and Gut-Derived Endotoxin in ALD

Ethanol modulates the expression and activity of several intracellular signaling molecules and transcription factors in monocytes and Kupffer cells. The recognition of pathogen-derived molecules, including endotoxin, occurs through pattern recognition receptors such as TLR expressed on Kupffer cells as well as on other cell types in the liver.[38,39] TLR-4 recognizes an endotoxin (LPS) with the cooperation of its co-receptors, CD14 or MD2, and LPS-binding protein (LBP) (**Fig. 3**). LBP is a soluble shuttle protein that directly binds LPS and facilitates the association between LPS and CD14. It is postulated that low concentrations of LBP enhance the LPS-induced activation of mononuclear cells, whereas the acute-phase rise in LBP concentrations inhibit LPS-induced immune cell activation.[40] The activation of macrophages, including Kupffer cells, by low concentrations of endotoxin (pg/ml to ng/ml range) depends on the expression level of the CD14 receptor. This receptor exists in two forms, membrane-associated CD14 (mCD14) and sCD14. CD14 recognizes extremely low levels of endotoxin in the presence of LBP, an acute phase reactant produced from hepatocytes. CD14 facilitates the transfer of LPS to the TLR-4/MD2 receptor complex and modulates the recognition of LPS. On the other hand, CD14 facilitates TLR-induced responses[38] and appears to be required for MyD88-independent signaling. MD2 is a soluble protein that non-covalently associates with TLR-4 and binds LPS directly to form a complex in the absence of TLR.[5]

Previous studies demonstrated that an inability to induce LPS signaling due to a deficiency of LBP, CD14, or mutation in TLR-4 protects mice from ALD.[5,41,42] Animal models of ALD have demonstrated increased levels of LBP and CD14,[43] and patients with alcoholic hepatitis have increased serum LBP levels. Thurman and colleagues have shown that elevated endotoxin levels after ethanol consumption trigger greater activation of Kupffer cells *via* enhanced CD14 expression in female rats.[44] The transcription factor NF-κB is a ubiquitous multiprotein complex that can be activated by a large number of extracellular stimuli such as cytokines, chemokines, growth factors, hypoxia reperfusion, stress-generated responses, and immune inflammatory cascades. NF-κB activation triggers the induction of inflammatory genes and may play a role in the initiation and progression of a variety of chronic inflammatory diseases. Therefore, it is noteworthy that NF-κB is activated in this process, leading to increases in TNF-α mRNA expression in the liver and more severe liver damage in females. Furthermore, increases in LBP and CD14 mRNA occur after only 24 to 48 h of ethanol feeding in rats, supporting the suggestion that these binding proteins play a role in liver damage instead of merely being a consequence of liver injury.[45] On the other hand, LBP plays an important role in early alcohol-induced liver injury by enhancing endotoxin-induced signal transduction, most

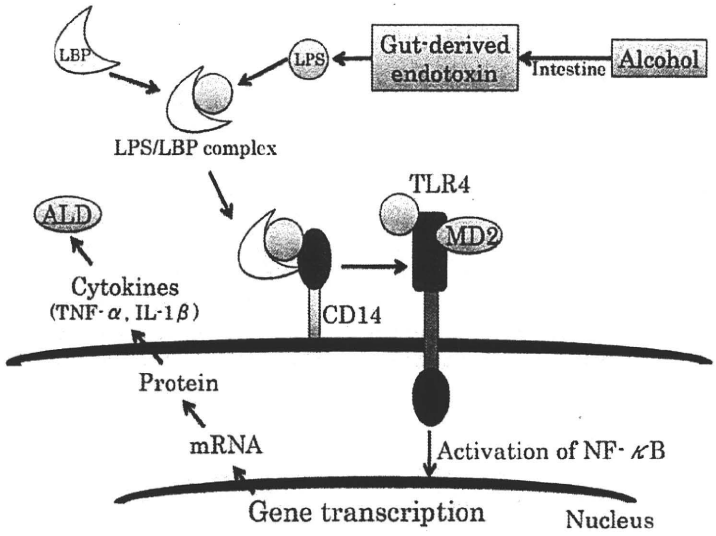

Fig. 3. Gut-derived endotoxin interacts with monocytes and the Toll-signaling pathway in acute alcohol administration
LPS is recognized by Toll-like receptors (TLR) expressed on Kupffer cells as well as on other cell types in the liver. LPS is recognized by TLR-4 and its coreceptors, CD14 and MD2, on Kupffer cells in the liver thus activating downstream signaling pathways culminating in the activation of transcription factors such as NF-κB. This process leads to increased inflammatory cytokine production in ALD. LPS, lipopolysaccharide; LBP, LPS-binding protein; TLR-4, Toll-like receptor-4; NF-κB, nuclear factor kappa B; ALD, alcoholic liver disease.

likely in Kupffer cells.[39] In addition, Uesugi *et al.*[46] showed that the C3H/HeJ strain carrying a mutation in the Toll/interleukin 1 receptor domain of the TLR-4 gene develops less severe early alcohol-induced liver injury than wild-type mice. In their animal model, the expression of functional TLR-4 is essential for alcohol-induced expression of TNF-α in the liver, which is responsible for hepatocyte injury *via* TNF-α receptor 1 (p55). Another study using animals in which the genes for CD14 were removed or which lacked LBP demonstrated that signaling processes mediated by endotoxin receptors were critical to the development of liver disease associated with chronic alcohol administration.[42] Recently, Mandrekar and Szabo[5] demonstrated that the recognition of LPS by TLR-4 on macrophages and other cell types in the liver, and activation of downstream signaling pathways culminating in the activation of transcription factors such as NF-κB and AP-1, lead to increased inflammatory cytokine production in ALD. In addition, LPS-induced mitogen-activated protein kinases (MAPKs) such as EPK and p38 also contribute to liver injury. In their review, Mandrekar and Szabo described the key signaling intermediates leading to alcohol-induced inflammation in ALD.

These reports have shown that mice lacking CD14 and mice lacking functional TLR-4 are protected against early alcohol-induced hepatotoxicity. These observations imply that receptors in LPS-sensing and/or downstream signaling pathways are important in the pathogenesis of ALD. It is, therefore, of interest that the activation of Kupffer cells by gut-derived endotoxin may occur *via* CD14 or TLR-4 pathways in ALD (**Figs. 2, 3**).

Pro-inflammatory Cytokine TNF-α and Alcoholic Hepatitis

Cytokines are pleiotropic regulatory peptides that can be produced by virtually every nucleated cell in the body, including most types of liver cells. Close temporal associations among Kupffer cell activation, increased transcription of TNF-α and related cytokine promoter genes, hepatic inflammation, and liver cell death have also been reported.[33] Furthermore, TNF-α plays a critical role not only in the production of inflammatory mediators, apoptosis, and necrosis, but also in cholestasis and fibrosis. Among the various cytokines, knowledge of the role of pro-inflammatory cytokine TNF-α in the pathogenesis of liver disease comes from studies of alcoholic and non-alcoholic steatohepatitis in animals. In combination with data from clinical studies, these findings indicate that TNF-α mediates not only the early stage of fatty liver disease but also the transition to a more advanced stage of liver damage.[47] On the other hand, close temporal associations among Kupffer cell activation, increased transcription of the genes for TNF-α and related cytokines, hepatic inflammation, and liver cell death have been reported in rodents with alcohol-induced steatohepatitis. In mice lacking TNF-α receptor 1 (TNF-R1, p55) or 2 (TNF-R2, p75), exposure to alcohol did not induce steatohepatitis *via* the TNF-R1 pathway.[36] In this animal model, it was also shown that free radicals act as redox signals for TNF-α production and do not directly damage cells in early alcohol-induced hepatic injury.[48] This constitutes good evidence that TNF-α is a key pathogenic

factor in alcohol-induced liver injury, regardless of the cause or source of TNF-α production. Thus, the involvement of TNF-α/NF-κB pathways is believed to play a key role in ALD, according to the evaluation of liver specimens taken from patients. As mentioned above, most authors now agree that activation of the innate immune system is a key event in the initiation of ALD and the disease's progression beyond the stage of fatty liver.[2,5,7,9,10,13] The research findings from McClain's laboratory have shown that activation of monocytes and macrophages with subsequent proinflammatory cytokine production plays an important role in certain metabolic complications of ALD and is a component of the liver injury of ALD.[9] Important elements of the innate immune system are phagocytes, such as macrophages and neutrophils, and macrophage-derived soluble mediators. The translocation of bacterial products from the intestinal lumen to the mesenteric circulation and its lymphatics induces regional and systemic production of TNF-α and other proinflammatory cytokines.[49] Increased circulating levels of cytokines have been postulated to cause many of the metabolic and nutritional abnormalities observed in ALD, especially in alcoholic hepatitis, and in more decompensated liver disease.[1]

Innate Immune System and Oxidative Stress in ALD

Gut-derived endotoxin and oxidative stress in ALD: Oxidative stress in the liver generates inflammatory cytokines (mainly TNF-α) and reactive metabolites of oxygen (mainly superoxides) that are largely released by sensitized and activated Kupffer cells. On the other hand, TNF-α administered intravenously with nanogram quantities of endotoxin has been reported to cause lethal shock, and it appears that TNF-α and endogenously produced endotoxin act synergistically in activating the complement system, which plays an important role in mediating tissue injury and lethality.[50–53] In addition, we have reported that the oxidative stress caused by TNF-α occurs as an enhancing effect of endotoxin or bacterial translocation from the intestinal gut with the reduction of RES function in various diseased states, and that TNF-α may cause a marked increase in the toxicity of oxidative stress by endotoxin.[52,54] In support of our findings, previous studies have indicated that endogenously produced endotoxin contributes to the extent of TNF-α hypersensitivity caused by D-galactosamine.[53] It is therefore possible that TNF-α production occurs as a result of bacterial or endotoxin translocation from the intestinal gut under conditions of reduced RES function in ALD. In addition, based on such studies, we hypothesize that increased production of ROS may prime or sensitize the liver to endotoxin/TNF-α, and such interaction may be important in alcohol-induced liver injury.[54] In fact, in the liver, Kupffer cells produce ROS in response to chronic alcohol exposure as well as to endotoxin. This is evidence that the direct interaction of NADH oxidase isozyme 4 with TLR-4 is involved in endotoxin-mediated ROS generation and NF-κB activation.[3] It is believed that chronic alcohol consumption primes the liver through sustained NF-κB activation and induction of basal and endotoxin-stimulated TNF-α expression. A possible role in modulating inflammatory processes associated with alcohol hepatotoxicity has been suggested by recent experiments using intragastric alcohol-fed rats with low circulating endotoxin levels.[55]

Recently, Vidali *et al.*[56] showed that immune responses to cell structures modified by alcohol-induced oxidative damage may fuel TNF-α production in patients who abuse alcohol and suggested a link between the generation of such immune responses and the promotion of liver disease in alcoholic patients.

Innate immune response and oxidative stress in ALD: The importance of ROS in the development of ALD is well documented. Oxidative stress-induced cellular responses play an important role in innate immune cell activation. As mentioned earlier, one consequence of Kupffer cell activation is the production of ROS, particularly superoxides, which in large amounts can lead to oxidative stress. Results of several studies suggest that the oxidative stress associated with chronic alcohol consumption is largely attributable to endotoxin-induced activation of Kupffer cells.[42,44,48] This hypothesis expanded the current assumption that alcohol-associated oxidative stress results primarily from the degradation of alcohol in the liver by a monooxygenase system called CYP2E1. Oxidative stress promotes inflammation, which is aggravated by an increase in pro-inflammatory cytokines. Several experiments using a variety of antioxidants and inhibitors of ROS-producing enzymes have explored the relationship of superoxide generation and oxidative stress with alcohol-induced liver injury.[44] Moreover, supplementation with antioxidant *in vitro* and *in vivo* has been shown to inhibit NF-κB activation and pro-inflammatory cytokine production in monocytes. Thus, inadequate levels of antioxidants may play a role in monocyte activation, and antioxidant therapy may represent a treatment option for ALD.[57]

Interestingly, in most studies using antioxidants, the alcohol-induced production of TNF-α was also reduced, suggesting that oxidative stress promotes TNF-α production. In addition, the activation of Kupffer cells by endotoxin *via* TLR-4 is involved in alcohol-induced liver injury, and alcohol-induced oxidative stress is important in the regulation of NF-κB and cytokine production in Kupffer cells.[9] This supports the suggestion that oxidative stress-sensitive transcription factor such as NF-κB may be potential sites of intervention in the treatment of inflammatory diseases, including AH.[57] Moreover, the findings from McClain's laboratory have shown that intravenous administration of the glutathione prodrug procysteine decreases cytokine production in stable alcoholic cirrhosis.[58] Yamashina *et al.*[59] have shown that interleukin-1 receptor-associated kinase (IRAK), one of the signaling molecules of

TLR-4, regulates tolerance and sensitization to endotoxin, and that acute alcohol increases IRAK expression through a mechanism dependent upon oxidant production. Further, NADPH oxidase plays a pivotal role in the increase in IRAK expression due to ethanol *via* activation of NF-κB signaling. These findings indicate that acute ethanol ingestion causes sensitization to endotoxin through mechanisms dependent upon oxidative stress. It is generally accepted that the activation of pro-inflammatory cytokines and other mediators plays an important role in the development of ALD.

Both chronic ethanol feeding and TNF-α cytotoxicity are associated with alterations of mitochondrial function. The mitochondria in TNF-α-exposed cells overproduce ROS derived from the respiratory chain. The mitochondria themselves then become the targets of ROS, thus setting up a cycle of injury. In addition to ROS production, TNF-α prompts the opening of the mitochondrial permeability transition (MPT). The MPT is the regulable opening of a large, nonspecific pore across the outer and inner mitochondrial membrane. Interestingly, ethanol may also increase susceptibility to MPT induced by TNF-α at the mitochondrial level, possibly through an increase in ROS production caused by respiratory chain dysfunction and/or CYP2E1.[47] Hence, the mechanisms affecting the interaction of ROS with inflammatory responses as well as alcohol-induced sensitization leading to hepatocyte death need further elucidation. It is widely accepted that ROS not only play a critical role in direct hepatocyte injury but also contribute to increased inflammatory responses contributing to liver damage.

CYP2E1-Induced Oxidative Stress and ALD

Modifications to the apolar side residues of membrane phosphoglycerides induced by active oxygen generation are thought to bring about structural changes in the membrane. Biomembranes and subcellular organelles are therefore the major sites of lipid peroxide damage. These peroxidative metabolic pathways are linked by a cytoplasmic glutathione (GSH) shuttle system, namely NAD(P)H oxidase and GSH peroxidase (GSH-Px). Furthermore, superoxide dismutase (SOD) may play an important role in protecting cells or tissues against the toxic effects of these superoxide radicals. Molecular oxygen itself is likely to be an important substrate for CYP2E1. CYP2E1, relative to several other P450 enzymes, displays high NADPH oxidase activity, as it seems to be poorly coupled with NADPH-cytochrome P450 reductase. From a toxicological point of view, interest in CYP2E1 revolves around the ability of this enzyme to metabolize and activate toxicologically important compounds such as ethanol, carbon tetrachloride, acetaminophen, benzene, halothane, and many other halogenated substrates. Interestingly, CYP2E1 expression was induced by ethanol with a corresponding 4- to 10-fold rise in mRNA levels in liver biopsy samples obtained from subjects who had recently drunk alcohol. Because CYP2E1 can generate ROS during its catalytic circle, and its levels are elevated by chronic treatment with ethanol, CYP2E1 has been suggested to be a major contributor to ethanol-induced oxidative stress and to ethanol-induced liver injury. Initial suggestions of a role for CYP2E1 in alcoholic liver injury arose from studies with an intragastric model of ethanol feeding in which prominent expression of CYP2E1 occurs and in which significant liver damage develops.[17–19,60] In these models, large increases in microsomal lipid peroxidation have been observed, and the ethanol-induced hepatic pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation.[60]

Hepatic alcohol oxidation: As noted above, alcohol-induced oxidative stress is linked to the metabolism of ethanol. Oxidation of ethanol mediated by ADH produces acetaldehyde, a toxic and reactive metabolite.[19,33,60,61] Three metabolic pathways for ethanol have so far been described in the human body, all involving the following enzymes: ADH, microsomal ethanol oxidizing system (MEOS), and catalase. **Figure 4** shows the ethanol and acetaldehyde oxidative reaction as illustrated in a review by Morita *et al.*[33] Each of these pathways could produce free radicals that affect the antioxidant system. The classical pathway of ethanol metabolism, which is catalyzation by ADH to form acetaldehyde, results in the formation of free radicals. In summary, the three pathways mainly generate acetaldehyde, which causes further liver injury and toxicity.

Progress in understanding the pathogenesis of ALD was achieved when it was discovered that alcohol affects the liver through not only nutritional disturbances but also direct toxicity because its predominant metabolism in the liver is associated with oxidation-reduction (redox) changes and oxidative stress. Redox changes are mediated by ADH, and oxidative stress is generated mainly by the activity of MEOS and its key enzyme CYP2E1, which releases free radicals.[19] Despite the discovery of CYP2E1 and its prevailing role in microsomal ethanol oxidation, the term MEOS was retained because cytochromes P450 other than CYP2E1 (such as CYP1A2 and CYP3A4)[62] may also contribute to ethanol metabolism in liver microsomes. Thus, the term MEOS characterizes total microsomal ethanol oxidation, not only that catalyzed by CYP2E1. Ethanol can enhance ROS formation through induction of CYP2E1 expression in the liver and in the brain. Further oxidation of acetaldehyde to form acetate is also accompanied by the generation of ROS.

CYP2E1 and oxidative liver injury in ALD: Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. One central pathway seems to be the induction of CYP2E1 expression. A working model of CYP2E1-dependent oxidative stress and toxicity is given in reviews by Cederbaum and colleagues (**Fig. 5**).[18,60] They described how the linkage between CYP2E1-dependent oxidative stress, stellate cell activation, mitochondrial injury, and GSH homeostasis contributes to

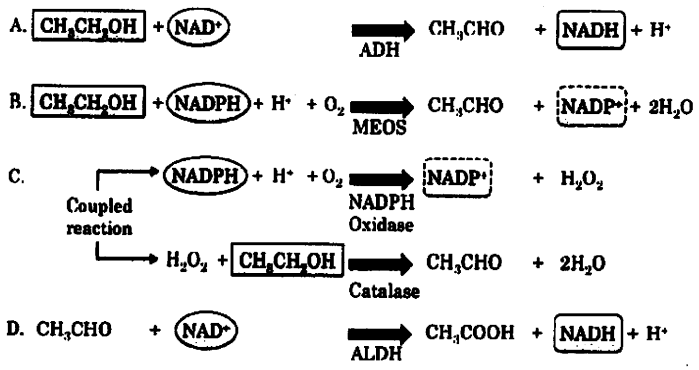

Fig. 4. Ethanol and acetaldehyde oxidation reaction
Ethanol is oxidized by (A) alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD+); (B) the hepatic microsomal ethanol-oxidizing system (MEOS), which involves CYP2E1 and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH); and (C) a combination of NADPH oxidase and catalase. Acetaldehyde is further oxidized to acetic acid by (D) aldehyde dehydrogenase (ALDH).

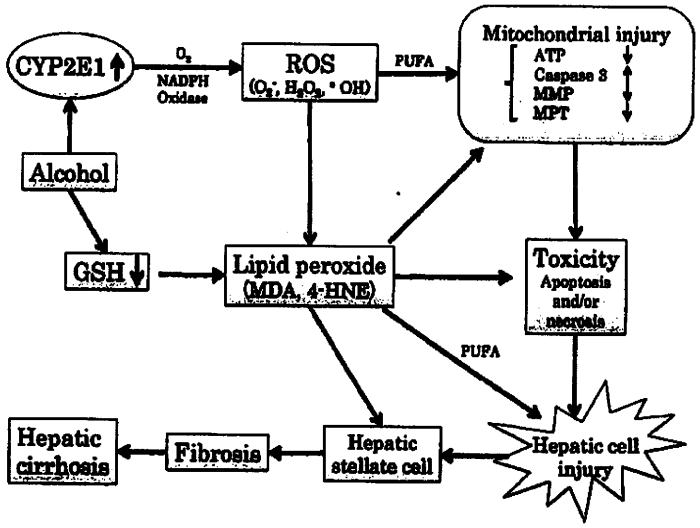

Fig. 5. CYP2E1-dependent oxidative stress and toxicity in alcoholic liver injury
Ethanol elevates CYP2E1 protein and activity levels by stabilizing the enzyme against proteasome-mediated degradation. CYP2E1, a loosely coupled enzyme, generates reactive oxygen species (ROS) during its catalytic cycle. These various ROS can cause cellular toxicity and damage to the mitochondria, especially in the presence of PUFA. In the presence of iron, the level of which is increased after ethanol treatment, more powerful oxidants, including the hydroxyl radical, appear. Lipid peroxide is postulated to be a key mediator of CYP2E1 toxicity and mitochondrial injury. A decrease in MMP and perhaps MPT causes the release of a pro-apoptotic factor, resulting in apoptosis. A decrease in ATP levels can cause necrosis. Some of the more stable CYP2E1-derived reactive species are diffusible and are postulated to exit hepatocytes and interact with other liver cells, such as hepatic stellate cells, to initiate a fibrotic response. MMP, mitochondrial membrane potential; MDA, malondialdehyde; HNE, 4-hydroxynonenal; PUFA, polyunsaturated fatty acid.

the toxic action of ethanol on the liver. CYP2E1 has very strong NADPH oxidase activity and extensively produces O_2^-, H_2O_2, and hydroxyethyl radicals that are likely to be responsible for the oxidative stress and lipid peroxidation caused by ethanol. It is of interest that some of the more stable CYP2E1-derived reactive species are diffusible and are postulated to exit the hepatocytes and interact with other liver cells, such as hepatic stellate cells, to initiate a fibrotic response. This free radical formation in biological systems in the presence of ethanol has been detected by spin trapping techniques.[63] A deterioration of liver mitochondrial function after chronic ethanol treatment is well documented. For example, the acetaldehyde produced by the oxidation of ethanol has toxic effects, inhibiting the repair of alkylated nucleoproteins, decreasing the activity of key enzymes, and markedly reducing oxygen use in mitochondria damaged by long-term ethanol consumption. Ethanol can also cause disruption between complex I and complex III of the

mitochondrial electron transport chain and enhance superoxide anion production.[64] It is noteworthy that the ethanol-induced oxidative damage to mitochondrial proteins not only diminishes mitochondrial function, but also promotes ROS production because of loss of protein activity in the mitochondrial electron respiratory chain. In addition, ethanol treatment induces changes in mitochondrial membrane structure that have been reported to account for defective mitochondrial uptake of GSH and a decline in the capacity to defend against oxidative stress.[65] Damage to mitochondria by CYP2E1-derived oxidants seems to be an early event in the overall pathway of cellular injury. Therefore, ethanol-induced oxidative stress is a result of the impairment of antioxidative defense and the production of ROS by the mitochondrial electron transport chain, alcohol-induced CYP2E1 expression: and activated phagocytes.[66] Indirectly, chronic ethanol consumption may augment oxidative stress by decreasing antioxidative defenses such as reductions in GSH-Px and GSH homeostasis. Supplementation with antioxidants has been shown to prevent ethanol-induced injury in liver, brain, heart, and skeletal muscle.[67] The end products of the peroxidation of polyunsaturated fatty acids, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), are used as markers to assess ROS-induced lipid peroxidation. Several other studies have suggested that ethanol may cause tissue damage through lipid peroxidation. Besides being specific markers of lipid peroxidation, both MDA and HNE have been implicated as mediators of diverse biologic effects and may play roles in the pathogenesis of alcoholic myopathy and liver disease. Oxidation of ethanol through the ADH pathway produces acetaldehyde, which is converted to acetate. Both reactions reduce NAD to its reduced form NADH. In an animal model, it was also shown that, in addition to the metabolic abnormalities caused by ADH activity, a new pathway of ethanol metabolism, namely MEOS, plays a key role in the progression of the disease. It is likely that several mechanisms contribute to alcohol-induced liver injury; the linkage between CYP2E1-dependent oxidative stress, mitochondrial injury, stellate cell activation, and GSH homeostasis may contribute to the toxic effect of ethanol on the liver.

Enhanced levels of hepatic CYP2E1 protein and mRNA were found in actively drinking patients. It is suggested that the most common cause of CYP2E1 expression is early alcoholic liver injury, such as alcoholic steatosis and alcoholic steatohepatitis.[17,19] In addition, as reviewed by Lieber,[19] liver disease in alcoholics is due not only to malnutrition but also to ethanol's hepatotoxicity linked to its metabolism by means of the ADH and CYP2E1 pathways and the resulting production of toxic acetaldehyde. Ethanol, the metabolites arising during its degradation, and novel compounds formed *via* ethanol-induced oxidative stress, especially during the action of ethanol-inducible microsomal CYP2E1, may, apart from directly damaging biological structures, affect signal transduction pathways thus modulating and potentiating damage.[68] As noted above, the induction of CYP2E1 expression by ethanol may play a role in the process by which ethanol generates a state of oxidative stress and in the mechanism responsible for alcoholic liver injury, although this is currently controversial.[17–19,60] To try to understand the basic effects and actions of CYP2E1, cell lines that constitutively express human CYP2E1 were developed. HepG2 cells are a human hepatoblastoma cell line that maintains several liver functions but does not express significant amounts of CYP2E1. HepG2 cell lines that overexpress CYP2E1 were established either by retroviral infection (MV2E1-9, or E9 cells) or by plasmid transfection (E47 cells). These cell lines have been used to study CYP2E1-catalyzed toxicity in alcohol-induced liver injury. Studies in Cederbaum laboratory with HepG2 cell lines expressing CYP2E1 showed that addition of ethanol, polyunsaturated fatty acids, or iron or depletion of GSH resulted in cell toxicity, increased oxidative stress, and mitochondrial damage, reactions that are prevented by antioxidants.[60] It seems that HepG2 cell lines overexpressing CYP2E1 may be a valuable model to characterize the biochemical and toxicological properties of CYP2E1. On the other hand, Bradford *et al.*,[69] using CYP2E1 and NADPH oxidase-knockout mice, concluded that CYP2E1 was required for ethanol induction of oxidative stress to DNA, whereas NADPH oxidase was required for ethanol-induced liver injury. In contrast, Thurman and colleagues suggest[70] that CYP2E1 may not play a role in alcohol liver injury, based on studies with gadolinium chloride and CYP2E1-knockout mice. They have presented powerful support for a role for endotoxin, the activation of Kupffer cells, and cytokines such as TNF-α in the ALD found with the intragastric infusion model.[35,36] As mentioned earlier, it is likely that several mechanisms contribute to alcohol-induced liver injury and that ethanol-induced oxidative stress is likely to arise from several sources, including CYP2E1, mitochondria, and Kupffer cells activated by gut-derived endotoxin.[60]

Pathophysiology in NAFLD and NASH

NAFLD is rapidly becoming a world-wide problem. NAFLD represents a spectrum of disease ranging from "simple steatosis," which is considered relatively benign, to NASH, NAFLD-associated cirrhosis, and end-stage liver disease. The term steatohepatitis followed by the underlying clinical condition if provided (*i.e.*, diabetes, obesity, hyperlipidemia, *etc.*) may be more appropriate to use in a histopathological diagnosis. Frequently associated with obesity, non-insulin-dependent diabetes mellitus, and hyperlipidemia, NAFLD is related to IR and oxidative stress as a critical pathogenic factor. Similarly, NASH is intimately related to IR syndrome, a constellation of disorders that result from abnormal production of hormones and cytokines that regulate inflammatory responses. The pathological picture resembles that of alcohol-induced liver injury, but

it occurs in patients who do not abuse alcohol. NAFLD is becoming the preferred term, and refers to a wide spectrum of liver damage, ranging from steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Steatohepatitis (NASH) represents only a stage within the spectrum of NAFLD. It should be differentiated from steatosis, with or without hepatitis, resulting from secondary causes, because these conditions have distinctly different pathogeneses and outcomes.[12]

It is now thought that NAFLD is a component of metabolic syndrome and type 2 diabetes that may progress to NASH in the long term, along with complications such as fibrosis and cirrhosis. Although the pathogenesis of NAFLD/NASH is not yet fully understood, much progress has been made in recent years in elucidating the mechanisms of progression from steatosis to more advanced liver inflammation and fibrosis. Distinguishing NAFLD from NASH is essential in the clinical setting, as NAFLD seems to have a benign course in the absence of co-existing liver disease, in sharp contrast to NASH, which is associated with increased morbidity and mortality and a reduced life expectancy. The pathology of the liver in alcoholic steatosis is remarkably similar to that of NAFLD, including NASH, suggesting some common pathogenic mechanism. Recently, there have been several excellent reviews[12,16,20–25,71] of the pathogenesis of NAFLD/NASH.

Cardiovascular risk factors for NAFLD: Patients with the primary forms of NAFLD/NASH, resulting from a multi-factorial process in which IR promotes the storage of fat in the liver, often present with various components of metabolic syndrome associated with an increased risk of cardiovascular morbidity and mortality. NAFLD is one of the most common causes of elevated levels of liver enzymes and chronic liver disease in Western countries. NAFLD is an emerging metabolic-related disorder characterized by fatty infiltration of the liver in the absence of alcohol consumption. NAFLD ranges from simple steatosis to NASH, which might progress to end-stage liver disease. In individuals with metabolic risk factors such as obesity, non-insulin-diabetes mellitus, hyperlipidemia, and IR, it is possible that alcohol consumed at lower quantities may promote hepatic steatosis. Patients with NAFLD frequently have many clinically significant co-morbidities (Table 1).[71] Obesity, type 2 diabetes, and hyperlipidemia are well known to co-exist with NAFLD, and it is important to systematically characterize them. The body mass index (BMI) and waist circumference should be measured to better characterize the degree (mild, moderate, and severe) and nature (central versus peripheral) of obesity. Type 2 diabetes and glucose intolerance occur very frequently in patients with NAFLD and have prognostic significance. In patients without preexisting type 2 diabetes, the presence of glucose intolerance and IR should be evaluated by obtaining fasting blood glucose, insulin levels, and hemoglobin A1c. Interestingly, NAFLD is closely linked to obesity, IR, and metabolic syndrome.[72,73] In addition, the presence of metabolic syndrome was significantly associated with IR. A central role for IR in the pathogenesis of NAFLD is supported by physiopathologic considerations, clinical associations, and laboratory investigations. Indeed, metabolic syndrome comprises a cluster of clinical and biochemical features, namely IR, glucose intolerance or diabetes, central obesity, hypertension, and dyslipidemia and is associated with significant cardiovascular morbidity and mortality. On the other hand, IR and metabolic syndrome lead to defective insulin-mediated inhibition lipolysis, mostly in visceral fat, while hyperinsulinemia results in increased hepatic synthesis of free fatty acids (FFAs) and decreased synthesis of apolipoprotein B-100, leading to triglyceride accumulation in the liver. Thus, IR results in both increased adipose tissue lipolysis and increased hepatic lipogenesis, leading to lipid accumulation in the hepatocytes, mainly in the form of triglycerides. Most importantly, NAFLD is characterized by the accumulation of triglycerides, which are formed from the esterification of FFAs and glycerol within the hepatocytes (Fig. 6).[24] It appears that NAFLD is the hepatic manifestation of metabolic syndrome and can vary from benign steatosis to end-stage liver disease.

Table 1. Co-morbidities commonly associated with NAFLD

Established conditions	Emerging conditions
Obesity	Obstructive sleep apnea
Type 2 diabetes/glucose intolerance	Hypothyroidism
Dyslipidemia	Obstructive sleep apnea
Metabolic syndrome	Hypopituitarism

Development of hepatocellular carcinoma from NAFLD/NASH: HCC accounts for the majority of primary cancers of the liver. More than 80% of HCC cases occur in less developed countries, particularly East Asia and sub-Saharan Africa, and are typically associated with chronic hepatitis B and C, although the incidence in these countries is decreasing.[74] One increasingly recognized cause of end-stage liver disease, cirrhosis, and HCC is NAFLD, particularly its more aggressive form, NASH. In fact, HCC secondary to NASH typically develops in the setting of cirrhosis, although cases of HCC arising in NASH without cirrhosis raise the possibility that carcinogenesis secondary to NAFLD can occur in the absence of advanced liver disease. The most common form of chronic liver disease in developed countries is NAFLD, which encompasses a clinicopathologic spectrum of diseases ranging from isolated hepatic steatosis to NASH, the more aggressive form of fatty liver disease that can progress to cirrhosis and complications such as hepatic failure and HCC.[16] In addition, the development of NASH is also associated with oxidative stress and the release of ROS, which likely contribute to the development of HCC. Obesity has already been linked to the development of primary liver cancer, and both obesity and diabetes mellitus

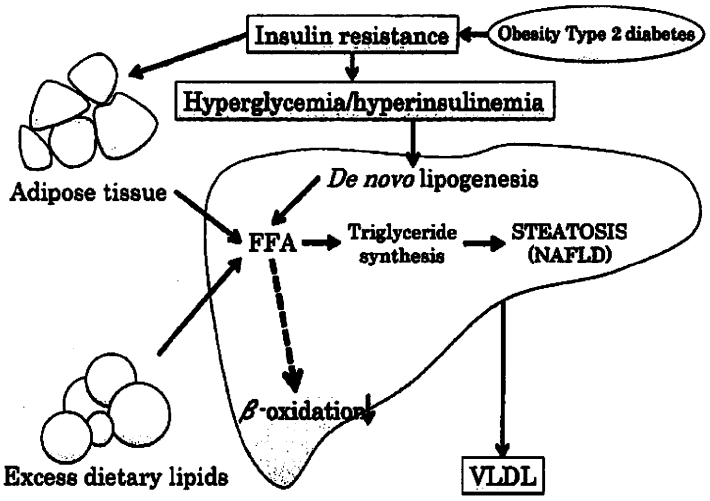

Fig. 6. Mechanisms of hepatic fat accumulation in NAFLD
FFAs arise in the liver from three distinct sources: lipolysis (the hydrolysis of FFA and glycerol from triglyceride) within adipose tissue, dietary sources, and *de novo* lipogenesis; VLDL, very low density lipoprotein.

have been closely correlated with increased risk of several malignancies, specifically HCC. The complications of NASH, including cirrhosis and HCC, are expected to increase in frequency with the growing epidemic of diabetes and obesity.

Progression from NAFLD to NASH: The most common cause of liver disease in developed countries is NAFLD, which includes NASH and associated complications. Understanding the mechanisms that lead to the progression from steatosis to advanced disease is clearly important to the design of treatment strategies directed at those who have developed progressive disease. Initial efforts to clarify the mechanisms that promote the progression from simple hepatic steatosis to steatohepatitis somewhat artificially divided disease mechanisms into "first and second hits."[75] This model considers the development of steatosis to be the "first hit," which increases the sensitivity of liver to the putative "second hit" that leads to the hepatocyte injury, inflammation, fibrosis, and cellular death characteristic of NASH.[76] Consistent with the hypothesis, the administration of endotoxin and pro-oxidants (second hit) resulted in significantly greater liver damage and lethality in obese mice with fatty liver compared to lean mice with healthy livers. Furthermore, in humans, the severity of steatosis is one of the strongest predictors of the development of NASH. The putative agents for the "second hit" were considered to be oxidative stress, associated CYP2E1 expression, cytokines (principally TNF-α), and gut-derived bacterial endotoxin (**Fig. 7**).[76] However, the initial and subsequent mechanisms may blend into each other. For example, the "second hit" that drives progression to steatohepatitis may be ongoing in individuals with steatosis, but these individuals are capable of compensating for the stress, thereby limiting disease progression.[77] The most widely accepted model to explain the progression from simple NAFLD to NASH is the "two-hit hypothesis" wherein fat accumulation per se is not sufficient to induce the progression to steatohepatitis, but renders the liver more susceptible to a "second hit" that, once imposed upon the steatotic liver, causes further aberrations that culminate in the development of NASH.[78]

Common Development and Progression of ALD and NAFLD/NASH

ALD is a common consequence of long-term alcohol abuse and a major cause of morbidity and mortality worldwide. Alcohol consumption is associated with a spectrum of diseases in the liver ranging from steatosis and steatohepatitis to cirrhosis and hepatocellular carcinoma. As noted above, alcoholic liver injury comprises interactions of various intracellular signaling events in the liver. Innate immune responses in the resident Kupffer cells of the liver, oxidative stress-induced activation of hepatocytes, fibrotic events in liver stellate cells, and activation of liver sinusoidal endothelial cells all contribute to alcoholic liver injury. However, some patients who are not regular drinkers can develop liver cirrhosis. Despite no history of patient alcohol intake, the histological findings of NASH are similar to those of alcoholic hepatitis. As described above, the "two-hit" theory,[75] has been proposed for the etiology of NASH, the underlying condition being simple steatosis, with the "first hit" being fatty degeneration as a result of IR, and the "second hit" being an aggravating factor such as inflammatory cytokines (including TNF-α), oxidative stress, or gut-derived endotoxin.

Oxidative stress as a common mechanism of alcoholic and non-alcoholic steatohepatitis pro-

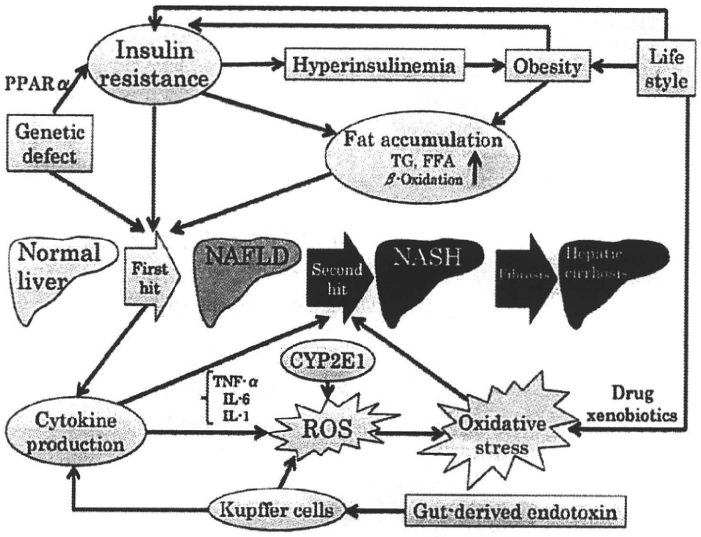

Fig. 7. Progression of NAFLD to NASH according to the "two-hit" theory and, ultimately, to liver cirrhosis.
This model considers the development of simple hepatic steatosis to be the "first hit" (insulin resistance, hyperinsulinemia, obesity, life style), thus increasing the sensitivity of the liver to the putative "second hit" (cytokine production, oxidative stress, gut-derived endotoxin) that leads to hepatocyte injury. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; TG, triglyceride; ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; PPARα, peroxisome proliferation-activator receptor-alpha.

gression: Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. In any event, the expression of CYP2E1 has been shown to play a key role in the pathogenesis of ALD, including alcoholic steatohepatitis, because of the oxidative stress generated.[17–19,60] This is significant not only from a mechanistic point of view but perhaps in terms of therapeutic treatment. If indeed CYP2E1-induced oxidative stress plays a central role in alcohol-induced liver damage, possible strategies for preventing this stress may be effective in attempts to minimize the hepatotoxicity of ethanol in humans. On the other hand, NASH causes steatosis, liver cell injury, inflammation, and variable necrosis. NASH is associated with obesity, type 2 diabetes, and hyperlipidemia, conditions in which CYP2E1 expression is induced.[60,71–73] Interestingly, hepatic CYP2E1 levels are increased in patients with NASH. In a recent study involving obese patients with nonalcoholic liver disease, increased CYP2E1 protein content and activity correlated with the development of liver injury.[79]

As noted above, although the pathogenesis of NAFLD and NASH has not yet been elucidated, a possible mechanism is provided by the "two-hit" theory,[75] with the "first hit" being the accumulation, due to several causes, of fatty acids in the liver. The "second hit" is the peroxidation of these fatty acids because of the oxidative stress produced by different factors such as CYP2E1 expression. Most of the data available from animal models and patients with NAFLD suggest that mitochondria are the most important intracellular source of ROS in NASH.[13,80] In NASH patients, at least 40% of mitochondria are structurally abnormal. The abnormalities consist of enlarged mitochondria and loss of mitochondrial cristae and paracrystalline inclusions, which are associated with impaired electron transport chain enzyme activity resulting in the uncoupling of oxidation from phosphorylation, leading to the further production of ROS.[13] Indeed, the oxidative stress caused by CYP2E1 and mitochondrial injury results in lipid peroxidation and membrane damage. Lieber[17,19] described how the damage caused by oxidative stress in both alcoholic and non-alcoholic steatohepatitis induces mitochondrial injury, which, in turn, exacerbates the oxidative stress. Mitochondrial damage is a key component of alcoholic liver injury, as established from mitochondrial structural defects. Mitochondrial dysfunction also contributes to the oxidative stress in NASH. Studies conducted with CYP2E1-overexpressing hepatocyte cell lines indicate potential links between CYP2E1-dependent oxidative stress, GSH homeostasis, and mitochondrial damage leading to cell death. It is therefore of interest that hepatic CYP2E1 expression is consistently upregulated in both clinical and experimental cases of NASH and that evidence of both oxidative stress and mitochondrial injury can be found.[13,19,80] CYP2E1 levels are invariably elevated in the liver of patients with NASH because fatty acids (which increase in obesity) and ketones (which increase in diabetes) are also substrates for CYP2E1; their excess up-regulates CYP2E1 expression. CYP2E1 leaks oxygen radicals, which, if they exceed the capacity of cellular defense systems, result in oxidative stress with pathologic consequences. This is true when excess alcohol has to be metabolized, as in alcoholic

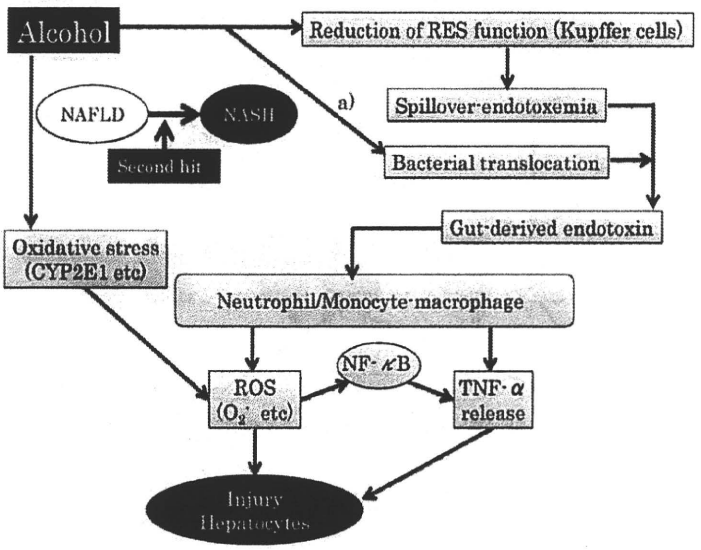

Fig. 8. Hypothetical schematic of common pathogenic mechanism in the liver injury caused by alcoholic or non-alcoholic steatohepatitis: role of gut-derived endotoxin, innate immune system, and oxidative stress
As noted above, the NAFLD/NASH model consideres the development of simple hepatic steatosis to be the "first hit," thus increasing the sensitivity of liver to the putative "second hit" that leads to hepatocyte injury. NAFLD, non-alcoholic fatty disease; NASH, non-alcoholic steatohepatitis; RES, reticuloendothelial system; ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; NF-κB, nuclear factor kappa B; [a]gastrointestinal tract.

steatohepatitis, or when CYP2E1 is confronted by an excess of ketones and fatty acids associated with diabetes, obesity, or both, resulting in NASH.[19] It is likely that CYP2E1 overexpression occurs in animals and humans with ALD and NASH. Interestingly, TNF-α has also been implicated as a critical mediator in ALD and NASH. The known role of oxidative stress in TNF-α cytotoxicity leads to the hypothesis that CYP2E1-induced oxidative stress may act to sensitize hepatocytes to death from TNF-α. It is therefore of interest that the ability of CYP2E1 overexpression to sensitize hepatocytes to TNF-α death receptor-mediated cytotoxicity may be a mechanism of liver cell injury and death in liver diseases such as alcoholic and nonalcoholic steatohepatitis in which CYP2E1 overexpression occurs.[60,81] Consequently, the damage from antioxidative activities in the blood of patients with NASH further indicates a role for free radical cytotoxicity in the pathophysiology of this disease.

Common immune response of alcoholic and non-alcoholic steatohepatitis: Growing evidence indicates that alcohol-induced oxidative modifications of hepatic constituents trigger specific immune responses and, in combination with genetic predisposition, lead to a breaking of self-tolerance in the liver.[66] The development of such adaptive immune responses likely favors the alcohol-mediated stimulation of the innate immunity system and, in turn, may contribute to hepatic inflammation during the evolution of ALD. In addition, intracellular mechanisms associated with LPS-induced signaling play a crucial role in the initiation and progression of ALD.[3,5] For example, LPS is recognized by TLR-4 on macrophages and other cell types in the liver, and the activation of downstream signaling pathways culminating in the activation of transcription factors such as NF-κB and AP-1 lead to increased inflammatory cytokine production in ALD. The histopathological findings in NASH are similar to those detected in alcoholic hepatitis, suggesting the existence of a common underlying mechanism. A possible mechanism is that increased endotoxin levels cause activation of Kupffer cells through TLR-4, leading to increased expression of TNF-α and increased levels of ROS. These events induce inflammation and fibrosis, progressing to NASH. Kawaratani *et al.*[82] focused on the innate immune reactivity of livers in rats with NASH, and found the overexpression of TNF-α, TLR-4, and CD14 in association with liver fibrosis and inflammation. In addition, TNF-α is overexpressed at the mRNA level both in adipose tissue and in liver, suggesting an important role for TNF-α in the pathogenesis of NASH. Indeed, LPS was found to up-regulate TNF-α production, thereby inducing hepatocyte apoptosis, in a murine NASH model. Endotoxin may play a key role in the pathogenesis of NASH. These results suggest that TLR-4 and CD14-mediated gut-derived endotoxin liver damage may also occur in NASH.

Conclusion

ALD is a common consequence of long-term alcohol abuse and a major cause of morbidity and mortality worldwide. It is well known that chronic ethanol ingestion

increases hepatotoxicity and produces fatty liver, hepatitis, and cirrhosis. In our present review, we would like to emphasize the important role of gut-derived bacterial toxins, the innate immune system, and oxidative stress in the development of steatohepatitis. **Figure 8** shows a hypothetical scheme for a common pathogenic mechanism in alcoholic and non-alcoholic steatohepatitis. The gut-liver axis, particularly gut-derived endotoxin, seems to play a crucial role in the pathogenesis of liver diseases caused by various insults, including alcohol. Not only inactivation of RES, which reduces the clearance of endotoxin, but also an increase in absorption of endotoxin from the intestine may be involved in ethanol-induced endotoxemia. On the other hand, activation of Kupffer cells by gut-derived endotoxin plays a pivotal role in ALD. Activated monocytes and macrophages have been postulated to play an important role in the pathogenesis of ALD. In the liver, Kupffer cells produce ROS in response to chronic alcohol exposure as well as exposure to endotoxin. Indeed, recent evidence shows that direct interaction of NADPH oxidase isozyme 4 with TLR-4 is involved in endotoxin-mediated ROS generation and NF-κB activation. Meanwhile, accumulating evidence suggests that alcohol-mediated upregulation of CYP2E1 expression may initiate lipid peroxidation *via* the production of ROS.

NAFLD/NASH is a liver disease characterized by histopathological features similar to those observed in ALD, but in the absence of significant alcohol consumption. The prevalence of NAFLD, including the more aggressive NASH, is increasing with growing epidemics of diabetes and obesity. IR and its subsequent inflammatory cascade that is associated with the development of NASH appear to play a significant role in the carcinogenesis of HCC. Although the pathogenesis of NAFLD/NASH has not yet been fully elucidated, a commonly cited mechanism is the "two-hit" theory. The most likely putative agents for the second hit were considered to be oxidative stress (CYP2E1 induction) and associated ROS and cytokines, principally TNF-α. These common pathogenic mechanisms may partly explain the development of progressive liver damage by alcoholic and non-alcoholic steatohepatitis. Some of the most definitive data on the importance of the innate immune system and oxidative stress in the pathogenesis of liver disease come from studies of alcoholic and non-alcoholic steatohepatitis in animals.

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Tumor Necrosis Factor-Alpha–Nuclear Factor-Kappa B-Signaling Enhances St2b2 Expression during 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Epidermal Hyperplasia

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The mouse cholesterol sulfotransferase St2b2 contributes to epidermal differentiation by biosynthesizing cholesterol sulfate (CS) from cholesterol in the epidermis. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) causes epidermal hyperplasia, an abnormal increase in epidermal cell numbers resulting from aberrant cell differentiation and an increase in St2b2 protein levels. The mechanisms underlying enhanced St2b2 expression and the pathophysiologic significance of the increased expression are unclear, however. To verify whether increased St2b2 levels are necessary for TPA-induced epidermal hyperplasia, the effects of St2b2-specific small hairpin RNA (St2b2-shRNA) on hyperplasia were examined in mice. St2b2-shRNA clearly suppressed TPA-induced epidermal hyperplasia and the expression of a marker of epidermal differentiation, involucrin (INV). Interestingly, treating mouse epidermal cells with tumor necrosis factor-alpha (TNFα) increased St2b2 expression. Furthermore, treatment with TNFα-siRNA or anti-TNF receptor antibodies reduced the TPA-induced enhancement of St2b2 expression. Treatment with BAY 11-7082, a specific inhibitor of nuclear factor-kappa B (NF-κB), diminished TPA-induced St2b2 expression. These results suggested that enhancement of St2b2 expression by TPA treatment occurs mainly through the TNFα–NF-κB inflammatory signaling pathway, which in turn leads to increased CS concentrations in epidermal cells and hyperplasia.

Key words cholesterol sulfate; epidermal differentiation; phorbol ester; nuclear factor-kappa B; sulfotransferase; tumor necrosis factor-alpha

Skin plays an essential role in protecting the body's internal environment against environmental insults. Keratinocyte differentiation, including expression of such cornified envelope proteins as involucrin (INV) and loricrin, is essential to maintain the structure and function of the epidermis. Disruption of the expression of these proteins causes epidermal dysplasia and dysfunction.[1,2]

Treating skin with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes epidermal hyperplasia through an abnormal increase in cornified envelope protein levels.[3] TPA is thought to produce these effects by inducing protein kinase C (PKC) activation, calcium influx, and release of inflammatory cytokines, for example.[4–6] The precise mediators of TPA signaling that cause epidermal hyperplasia, however, remain unclear.

Cholesterol sulfate (CS) is a chemical mediator that maintains epidermal homeostasis. Accumulation of CS—for instance, owning to a deficiency in the catabolic enzyme steroid sulfatase (SSase)—causes dyskeratosis.[7–9] Levels of the CS biosynthetic enzyme cholesterol sulfotransferase (Ch-ST) are also associated with the extent of epidermal differentiation. In particular, St2b2, a member of the cytosolic sulfotransferase family, is the primary Ch-ST in mouse epidermis,[10] where it plays a role in the expression of INV.[11] Ch-ST activity increases together with cornified envelope protein levels after TPA treatment.[12,13] Thus, St2b2 may be linked to TPA-induced epidermal hyperplasia.

Expressed beginning at early cornification, INV is involved in construction of the cornified envelope.[14,15] Overexpression of INV distorts the structure of the epidermis.[1,16] Furthermore, cells that express INV in response to various stimuli are selectively expelled from the basal layer composed of uncornified epidermal cells.[17] Thus, INV expression levels can be used as a marker of the extent of epidermal differentiation.

Application of TPA on mouse skin causes an intense inflammatory response. The associated signaling is mediated by various cytokines and regulatory factors, such as tumor necrosis factor-alpha (TNFα) and interleukins, which are involved in epidermal differentiation and dyskeratosis.[18,19] Indeed, TNFα levels increase when epidermis is exposed to TPA.[20,21] TNFα binding to TNF receptor (TNFR) results in activation of a number of transcription factors, including nuclear factor-kappa B (NF-κB). Moreover, treating keratinocytes with anti-inflammatory drugs suppresses TPA-induced increases in Ch-ST activity,[13,22] suggesting that TNFα–NF-κB signaling mediates TPA-induced enhancement of St2b2 expression.

The present study shows that TNFα–NF-κB signaling contributes to TPA-induced epidermal hyperplasia *via* increased St2b2 expression.

MATERIALS AND METHODS

Materials Cholesterol, CS, 3′-phosphoadenosine-5′-phos-

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phosulfate (PAPS), TPA, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). [^{35}S]-PAPS and [^{14}C]-cholesterol was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, U.S.A.). PKCη pseudosubstrate inhibitor was purchased from Merck (Darmstadt, Germany). Recombinant mouse TNFα and mouse TNF enzyme-linked immunosorbent assay (ELISA) Kit 2 were purchased from Becton Dickinson Co. (San Diego, CA, U.S.A.). TNFα-small interfering RNA (siRNA), control-siRNA A, siRNA transfection medium, and siRNA transfection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-mouse TNFR hamster monoclonal antibodies (TNFR-Ab) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Hamster IgG (LEAF^{TM} purified American hamster IgG isotype control Clone) was purchased from BioLegend (San Diego, CA, U.S.A.). BAY 11-7082 was purchased from BIOMOL Research Labs (Plymouth, PA, U.S.A.). The St2b2-specific small hairpin RNA (St2b2-shRNA)-expressing adenovirus AdSt2b2-shRNA was constructed previously.[11] All other chemicals used were of the highest grade available.

Animal Treatments Six-week-old female CD-1 mice (Charles River Japan, Atsugi, Japan) were housed in an air-conditioned room (22—23 °C) with a 12-h light period from 6 a.m. to 6 p.m. Food and water were available *ad libitum*. We used female mice in this study because epidermal St2b2 levels and cholesterol sulfation are slightly higher in females than in males.[10,11] Animal experiments were conducted in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology of Japan. A dorsal area (2 cm^2) of each 6-week-old mouse was shaved using an electric shaver 24 h before the first treatment.

TPA treatment: TPA (16 nmol) dissolved in 200 μl of acetone (vehicle) was painted onto a shaved dorsal area of each mouse using a pipette. The skin was removed 40 h after treatment.

AdSt2b2-shRNA and TPA treatment: A previous report showed that a target gene could be introduced into epidermal cells by painting a mouse's skin with a suspension containing gene-encoding adenovirus.[11] Therefore, the shaved area of the mice was painted with a suspension containing 1.0×10^7 $TCID_{50}$ (50% titer culture infectious dose) of AdSt2b2-shRNA or control adenovirus (AdControl) 24 h before TPA treatment. TPA treatment was performed as described above.

Cell Treatments Neonatal mouse epidermal (NME) cells were prepared from newborn CD-1 mice, born to pregnant CD-1 mice (Charles River Japan, Atsugi, Japan), based on a previously reported method.[11] The cells were plated onto 100-mm dishes (treatment with AdSt2b2-shRNA and TPA) or 6-well plates (other experiments) at 3—4×10^5 cell/cm^2 and cultured in defined keratinocyte serum-free medium (SFM; 100-mm dishes, 10 ml; 6-well plates, 2 ml) (Invitrogen, Carlsbad, CA, U.S.A.). The first treatment was performed after 24 h of culture.

TPA treatment: NME cells were cultured in defined keratinocyte SFM containing 10 nM TPA or 0.1% (v/v) dimethyl sulfoxide (DMSO) (vehicle). Cells were harvested 0, 3, 6, 12, 24, or 40 h after TPA treatment.

AdSt2b2-shRNA and TPA treatment: NME cells were infected with AdSt2b2-shRNA (multiplicity of infection (MOI): 0, 1, 3, or 6) 24 h before TPA treatment. The total amount of adenovirus was adjusted to an MOI of 6 with AdControl. Cells were further cultivated in defined keratinocyte SFM containing 10 nM TPA or 0.1% (v/v) DMSO (vehicle) for 40 h.

PKC inhibitor and TPA treatment: The PKC inhibitor PKCη pseudosubstrate inhibitor was diluted to 0.5 or 1.5 mM with phosphate buffered saline (PBS) (vehicle). NME cells were incubated with 0.5 or 1.5 μM PKC inhibitor for 1 h. TPA (10 nM) treatment was performed as described above.

TNFα Treatment: Recombinant mouse TNFα was diluted to 10, 30, or 100 ng/ml with PBS containing 2 mg/ml BSA (vehicle). NME cells were cultivated in defined keratinocyte SFM containing TNFα (0, 10, 30, or 100 pg/ml) for 24 h.

AdSt2b2-shRNA and TNFα Treatment: NME cells were infected with AdSt2b2-shRNA or AdControl (MOI: 6) 24 h before TNFα treatment. Treatment with 30 pg/ml TNFα was carried out as described above.

TNFα-siRNA and TPA Treatment: Twenty-four hours before TPA treatment, NME cells were treated with TNFα-siRNA at 0, 15, 45, or 90 nM using siRNA transfection medium and siRNA transfection reagent. The concentration of siRNA was adjusted to 90 nM with Control-siRNA A. TPA (10 nM) treatment was performed as described above.

TNFR-Ab and TPA Treatment: NME cells were incubated with 0, 0.5, 2, or 8 μg/ml TNFR-Ab for 6 h. The total concentration of antibody was adjusted to 8 μg/ml with hamster IgG. TPA (10 nM) treatment was performed as described above.

NF-κB Inhibitor and TNFα Treatment: The NF-κB inhibitor BAY 11-7082 was dissolved to 1, 3, or 10 mM in DMSO (vehicle). NME cells were incubated with 0, 1, 3, or 10 μM BAY 11-7082 for 1 h. TNFα (30 pg/ml) treatment was performed as described above.

Preparation of Cytosolic, Microsomal, and Membrane Fractions Skin was removed and the dermis was excised from the skin by scraping with a surgical razor. The epidermis was homogenized in homogenizing buffer containing 75 mM potassium phosphate (pH 7.4), 75 mM KCl, and 1 mM dithiothreitol using a Polytron PT-10 homogenizer at 4 °C. The homogenate was filtered through gauze and centrifuged for 20 min at 4 °C and 9000×*g*. The resultant pellet was resuspended in homogenizing buffer and used as the membrane fraction. The supernatant (S-9) was further centrifuged for 60 min at 4 °C and 105000×*g*. The resultant supernatant was used as the cytosolic fraction, and the pellet was resuspended in homogenizing buffer and used as the microsomal fraction. NME cells were harvested and homogenized using homogenizing buffer. The homogenate was fractionated using the same method described for fractionation of epidermis. Protein concentrations were determined using the Bradford method.[23]

Immunoblotting Immunoblotting for St2b2 and INV was performed as described previously.[11] Cytosolic samples (20—40 μg of protein/lane) and membrane fractions (10—20 μg of protein/lane) were loaded.

Determination of Enzymatic Activities Cholesterol-sulfating activities were determined using a previously reported method.[11] The assay for CS desulfation was performed as described previously with some modifications.[24] SSase activity was determined based on desulfo-conjugation

Table 1. Primer Sequences for RT-PCRs

Gene		Sequence
St2b2	Sense	5′-TGTGGAGCTCGTCTGAGAAAAATGTT-3′
	Antisense	5′-TTGAAGGCGCTTATGATGGTCTCGC-3′
St2a4/9	Sense	5′-TGATGTCAGACTATAATTGGTTTGAAGGC-3′
	Antisense	5′-GGTTATGAGTCGTGGTCCTTCCTTATTG-3′
SSase	Sense	5′-GACGCTCGTCTACTTCACCTC-3′
	Antisense	5′-CTCCCAGTTGTTGCCCTTCC-3′
GAPDH	Sense	5′-TGCATCCTGCACCACCAACTG-3′
	Antisense	5′-GTCCACCACCCTGTTGCTGTAG-3′

of [^{14}C]-CS, which was prepared using [^{14}C]-cholesterol as a substrate, PAPS as a sulfate donor, and recombinant St2b2 protein, and purified using thin layer chromatography (TLC aluminum plate silica gel 60, Merck, Darmstadt, Germany). The desulfation assay was performed in 30 mM Tris–HCl buffer (pH 7.2) containing 0.25 M sucrose, 20 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 3 μM [^{14}C]-CS, and microsomal protein (100 μg) in a volume of 10 μl. The mixture was incubated at 37 °C for 12 h, and the reaction was terminated with 5 μl of acetonitrile. Ten-microliter aliquots of the reaction mixture were applied to a TLC plate. Metabolites on the chromatogram were developed using a solvent system of ethyl acetate/methanol/water (80 : 20 : 7.5 by vol). Radioactive spots were analyzed using a FLA-3000 image analyzer (Fujifilm Corporation, Tokyo, Japan).

Quantification of CS CS levels in epidermis and in NME cells were quantified as reported previously.[11]

Isolation of Total RNA and Reverse Transcription-Polymerase Chain Reactions (RT-PCRs) Total RNA was prepared from epidermis tissue or NME cells using the acid guanidinium thiocyanate–phenol–chloroform method. mRNA levels were measured using RT-PCRs. The reaction conditions included 30 cycles of a three-phase PCR (denaturation at 95 °C for 15 s; annealing at 55 °C for 30 s; extension at 72 °C for 30 s). PCR products were analyzed on 2% (w/v) agarose gels containing ethidium bromide. The intensities of the stained bands were measured using NIH image software (version 1.59, Bethesda, MD, U.S.A.). The specific primers used for the PCRs are shown in Table 1.

Immunohistochemistry Dorsal skin was frozen in OCT Compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan). Specimens were sectioned using a cryostat and stained with methyl green-pyronin.

Detection of Intracellular and Extracellular TNFα Concentrations TNFα levels in the cells (S-9 fraction) and media were quantified using Mouse TNF ELISA Kit 2 in accordance with the manufacturer's instructions.

Statistical Analysis All data are shown as the means± S.D. Statistical differences between groups were assessed by one-way ANOVA followed by Tukey's multiple comparison. Probability values less than 0.05 were considered statistically significant.

RESULTS

Effects of TPA Treatment on the Activities of Ch-ST and SSase in Mouse Skin To clarify the contributions of Ch-ST and SSase to the increased CS concentrations after TPA treatment, CS levels in skin, the specific activities of

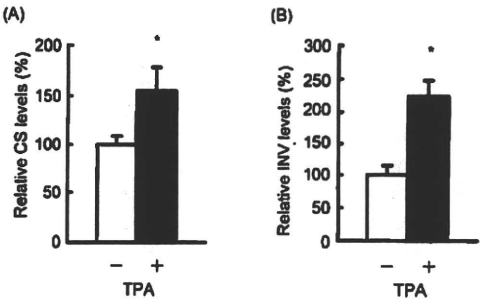

Fig. 1. Expression of CS and INV Protein in TPA-Treated Mouse Epidermis

TPA (16 nmol) dissolved in acetone (vehicle) was painted on shaved dorsal skin in mice. The skin was removed 40 h after treatment, and the dermis was excised. (A) The amount of CS in the epidermis was measured using thin layer chromatography. (B) INV levels in the membrane fraction from the epidermis were determined by immunoblotting. Expression levels are shown as ratios to levels observed in control TPA(−) epidermis. Data are shown as the means±S.D. (n=3). * p<0.05.

Table 2. Effects of TPA Treatment on Ch-ST and SSase Activities in Mouse Epidermis

Activity	Vehicle	TPA
Ch-ST (pmol/mg protein/min)	2.64±0.02	3.82±0.51*
SSase (pmol/mg protein/min)	0.69±0.11	0.78±0.08

Cytosolic protein and microsomal protein were examined from the epidermis used in Fig. 1. Methods used to determine the activities of Ch-ST in the cytosol and SSase in the microsomal fraction are provided in the Materials and Methods. Each value is shown as the mean±S.D. (n=3). * p<0.05.

Ch-ST and SSase, and St2b2, St2a4/9, and SSase mRNA levels were determined. Skin INV protein levels were used as a marker of epidermal differentiation. After TPA treatment, CS levels in mouse epidermis were 50% higher than those in control TPA(−) skin (Fig. 1A). TPA treatment also enhanced INV expression (120% increase; Fig. 1B) and skin Ch-ST activity (40% increase; Table 2). St2b2 mRNA and protein levels in skin increased by 120% and 50%, respectively (Fig. 2). Neither the activity nor mRNA levels of SSase changed significantly, however (Fig. 2, Table 2). In addition, mRNA encoding St2a4 or St2a9, which mediate cholesterol sulfation in mice, was not detected in normal mouse epidermal cells, as reported previously,[10] or in TPA-treated skin (Fig. 2).

Effects of AdSt2b2-shRNA on TPA-Induced Epidermal Differentiation in NME Cells St2b2-shRNA was then administered to NME cells and the levels of CS and INV protein were examined (Fig. 3). St2b2 protein levels increased by 40% after TPA treatment in control AdSt2b2-shRNA(−) cells, whereas they MOI-dependently decreased in response to AdSt2b2-shRNA. St2b2 expression decreased to 80% of that observed in control TPA(−) cells (Fig. 3A). Consistent with this finding, CS levels also decreased to 60% of those observed in control cells following infection with AdSt2b2-shRNA (MOI, 6; Fig. 3B). A correlation was observed between the levels of CS and St2b2 protein (Fig. 3D). The TPA-mediated increase in INV expression levels was attenuated to 90% of levels detected in control cells following infection with AdSt2b2-shRNA (MOI, 6: Fig. 3C). A correlation was observed between expression levels of INV and St2b2 (Fig. 3E). SSase mRNA levels were not

affected by infection with AdSt2b2-shRNA (data not shown).

Effects of a PKC Inhibitor on TPA-Induced Increases in St2b2 and INV Expression in NME Cells The effects of a PKC inhibitor on St2b2 and INV protein levels were then examined during TPA-induced epidermal differentiation in NME cells. After TPA treatment, St2b2 protein levels increased to 160% of those observed in control cells (PKC inhibitor(−) and TPA(−)); the PKC inhibitor (0.5 or 1.5 μM) did not significantly affect St2b2 levels (Fig. 4A). Expression levels of INV protein also increased to 160% of those observed in control cells after TPA treatment, whereas, in response to 1.5 μM PKC inhibitor, the level decreased to 70% of that detected in control cells (Fig. 4B).

Knocking Down St2b2 Gene Expression during TPA-Induced Epidermal Hyperplasia in Mouse Skin The effects of knocking down St2b2 gene expression were then examined during epidermal hyperplasia. As shown in Fig. 5, the thickness of the epidermis increased after TPA treatment, but not when AdSt2b2-shRNA was also introduced together with TPA. We also examined Ch-ST activity and expression

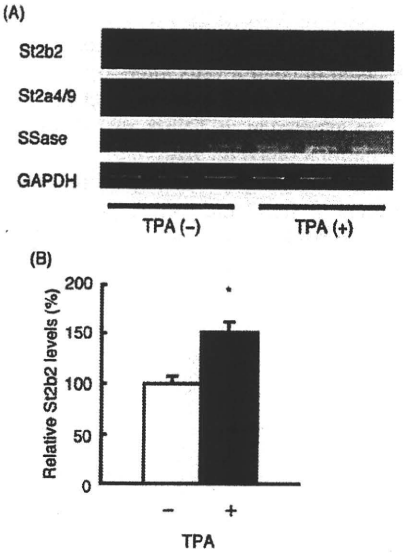

Fig. 2. Expression of St2b2, St2a4/9, and SSase mRNA and St2b2 Protein in Mouse Epidermis after Treatment with TPA

Total RNA and cytosolic protein were prepared from the epidermis used in Fig. 1. (A) mRNA levels were measured using RT-PCRs. (B) St2b2 protein levels in the cytosol were determined by immunoblotting. Expression levels are shown as ratios to those in control TPA(−) epidermis. Data are shown as the means±S.D. (n=3). * p<0.05.

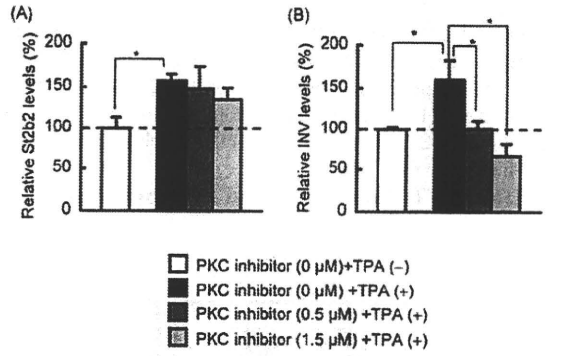

Fig. 4. Effects of PKC Inhibitor Treatment on St2b2 and INV Expression in NME Cells

NME cells (3×10^5 cells/cm^2) were incubated with PKCη pseudosubstrate (0.5 or 1.5 μM) for 1 h, and then cultivated with 10 nM TPA or 0.1% (v/v) DMSO (vehicle) for 24 h. Levels of St2b2 protein in the cytosol (A) and INV protein in the membrane fraction (B) were determined by immunoblotting. Expression levels are shown as ratios to those in control PKC inhibitor(−) and TPA(−) cells. Data are shown as means±S.D. (n=3). * p<0.05.

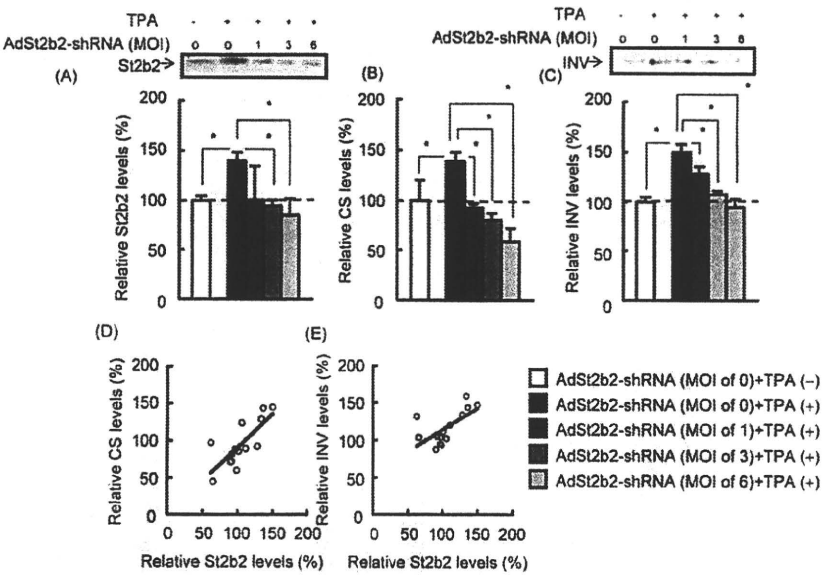

Fig. 3. Effects of AdSt2b2-shRNA Infection on TPA-Induced Epidermal Differentiation in NME Cells

NME cells (3×10^5 cells/cm^2) were infected with AdSt2b2-shRNA (MOI: 1, 3, or 6) 24 h before TPA treatment. The total amount of adenovirus was adjusted to an MOI of 6 using AdControl. Cells were then treated with 10 nM TPA or 0.1% (v/v) DMSO (vehicle), and harvested 40 h later. The cells were subjected to the following analyses. (A) Expression of St2b2. Example immunoblotting of St2b2 in the cytosol is shown above the graph. (B) CS levels were quantified using thin layer chromatography. (C) Expression of INV. Example immunoblotting of INV in the membrane fraction above the graph. (D) Correlation between St2b2 expression and CS levels. (E) Correlation between protein expression of St2b2 and INV. Expression levels are shown as ratios to those observed in control AdSt2b2-shRNA(−) and TPA(−) cells. Data are shown as the means±S.D. (n=3). * p<0.05.