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Activation of mitogen activated protein kinase (MAPK) during D-galactosamine intoxication in the rat liver

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Abstract—A significant increase in plasma glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase was observed 6 h after intraperitoneal administration of D-galactosamine (D-Galn). Three hours after administration of D-Galn, the vitamin C concentration in the liver decreased significantly compared to that in a control group and thereafter the hepatic vitamin C concentration remained at a significantly lower level. Phosphorylated JNK (c-Jun NH_2-terminal kinase) and phosphorylated ERK (extracellular signal-regulated kinase) started increasing 3 h after D-Galn treatment and remained at a high level for 6–12 h after the treatment, while phosphorylated p38 MAPK increased significantly 6 h after D-Galn administration. These results indicated that oxidative stress and the activation of JNK and ERK took place almost simultaneously, followed by the activation of p38 MAPK.

D-Galactosamine (D-Galn) is known as a toxin causing necrosis of the liver by UTP depletion and inhibition of protein synthesis.[1] Recently, we reported that radical reactions were caused by D-Galn in the early stage based on a decrease in vitamin C,[2] which was first consumed by oxidative stress and also was the most sensitive indicator of oxidative stress.[3] In the later stage, extensive radical reactions took place evidenced by a significant increase in lipid hydroperoxides,[2] which were measured by a specific and sensitive method involving chemical conversion of lipid hydroperoxides into aromatic phosphine oxide, followed by the measurement of oxide by HPLC.[4]

Although D-Galn has long been known as a typical hepatotoxin causing necrosis,[1] D-Galn was shown to induce apoptosis involving the activation of caspase-3 in the rat liver based on histochemical observations [in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method].[2] These studies demonstrate that D-Galn causes liver cell necrosis as well as apoptosis through oxidative stress, which is shared by many environmental toxicants. In addition, D-Galn increased the plasma concentration of ceramides,[5] which were a possible candidate to induce damages of extrahepatic tissues during fulminant hepatic failure.

In this study, early changes leading to liver cell death caused by D-Galn were investigated with special reference to the activations of mitogen activated protein kinase (MAPK) including JNK (c-Jun NH_2-terminal kinase), p38 MAPK (mitogen activated protein kinase), and ERK (extracellular signal-regulated kinase). These MAPKs have been investigated extensively in cultured cells but a study of MAPK in animal tissues has been made scarcely. In addition, MAPKs are assumed to be activated by oxidative stress in cultured cells. However, their relationship has never been studied in the liver. In this study, time courses of oxidative stress and MAPK activation were compared for the first time in the liver based on phosphorylation profiles of MAPKs and the level of vitamin C, the most sensitive indicator of oxidative stress.[3]

D-Galn (1 g/kg body weight) was intraperitoneally administered to rats.[6] After 1.5 and 3 h, the activity of plasma GOT and GPT did not significantly differ from

Abbreviations: ERK, extracellular signal-regulated kinase; D-Galn, D-galactosamine; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen activated protein kinase.

Keywords: ERK, galactosamine; JNK; MAPK; Oxidative stress; p38; Vitamin C.

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Table 1. The plasma GOT and GTP (Karmen units), and the vitamin C concentration (nmol/g liver) in D-galactosamine-treated rats after 1.5, 3, 6, 12, and 24 h, and control rats

	Control	1.5 h	3 h	6 h	12 h	24 h
GOT	69.2 ± 13.2	52.3 ± 23.8	124 ± 22	517 ± 227*	793 ± 223*	2560 ± 538*
GPT	24.2 ± 2.8	25.8 ± 14.9	49.7 ± 4.5	295 ± 104*	426 ± 116*	1519 ± 308*
Vitamin C	1498 ± 106	1571 ± 219	1137 ± 199*	1118 ± 114*	1104 ± 225*	978 ± 49*

D-Galactosamine (1 g/kg body weight) was intraperitoneally administered to rats. After 1.5, 3, 6, 12, and 24 h, plasma GOT and GPT, and the liver vitamin C concentration were determined as described in the text. Control rats received saline and determinations were made after 3 h. Values are means ± SD for 4–6 rats and asterisks indicate significant differences from the control group (ANOVA Fisher's protected least significant difference test (PLSD), $*P < 0.01$.

that of the control group, which was administered saline (Table 1).[7] After 6 h, plasma GOT and GPT were significantly higher than that of the control group (Table 1). Activities of both enzymes continued increasing and after 24 h, they augmented to an extremely high level compared to those of the control group (Table 1) consistent with a previous study.[2] These results showed that the necrotic process was already initiated approximately 6 h after D-Galn administration and was enhanced thereafter. It was also shown that apoptosis involving the activation of caspase-3 took place 18 h after D-Galn injection.[2]

The liver concentration of vitamin C was not affected at 1.5 h, but was significantly decreased 3 h after D-Galn injection, when necrosis was not appreciably induced based on plasma GOT and GPT (Table 1). Thereafter, the hepatic vitamin C concentration remained at a significantly lower level than that in the control. These results indicated that oxidative stress was significantly enhanced as early as 3 h after D-Galn administration.

Phosphorylated JNK at 54 kDa significantly increased 3 h after treatment with D-Galn, when oxidative stress in the liver had been significantly enhanced as described above, while the JNK protein level was almost the same as that of the control from 1.5 to 24 h after D-Galn treatment (Figs. 1 and 2).[8] The phospho-JNK level remained at a high level for 6–12 h after D-Galn treatment and declined thereafter (Figs. 1 and 2A).

Phosphorylated ERK at 42 kDa was significantly increased after 3 h and maintained a significantly high level for 6–24 h after D-Galn injection, while the level of ERK1 and ERK2 proteins was almost the same as that of the control from 1.5 to 24 h after D-Galn treatment (Figs. 1 and 2B).

Phosphorylated p38 MAPK was significantly increased 6 h after administration of D-Galn, while the level of p38 MAPK protein was almost the same as that of the control from 1.5 to 24 h after D-Galn treatment (Figs. 1 and 2C). These results indicated that oxidative stress and the phosphorylation of JNK and ERK were almost simultaneously increased approximately 3 h after administration of D-Galn, followed by the transient activation of p38 MAPK.

Proteins comprising the MAPK family are important mediators of signal transduction processes that serve to regulate diverse cellular responses to extracellular

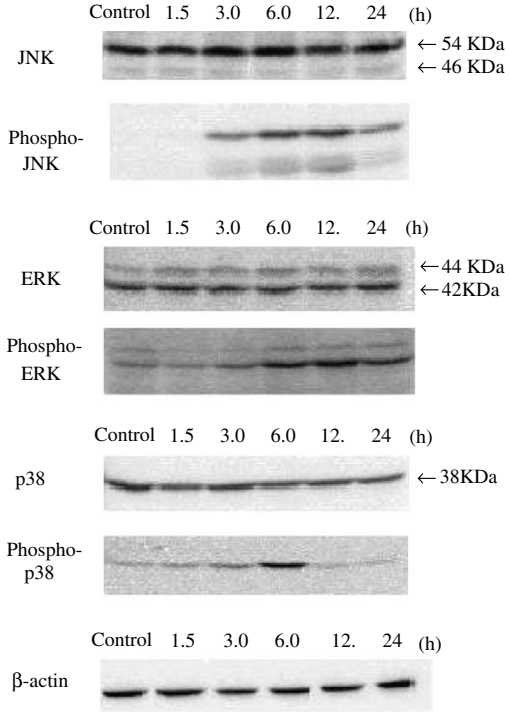

Figure 1. MAPK and phospho-MAPK in the livers of rats 1.5, 3, 6, 12, and 24 h after D-galactosamine injections and control rats. D-Galactosamine (1 g/kg body weight) was intraperitoneally administered to rats. After 1.5, 3, 6, 12, and 24 h, protein concentrations of MAPK and phosphorylated MAPK (described as p-ERK, p-p38, and p-JNK) in the livers were determined as described in the text. Three hours after the administration of saline, determinations were made for control rats.

stimuli. The three major subclasses of the MAPK family are JNK, p38 MAPK, and ERK. Among them, JNK was shown to be activated in the induction of hepatocyte death by TGF-β1,[9,10] ischemia–reperfusion,[11] thioacetamide,[12] oxidative stress induced by menadione,[13] and carbon tetrachloride.[14] The present study also indicated that activation of JNK occurred before apoptosis involving activation of caspase-3 and widespread necrosis of liver cells induced by D-Galn.[2] An increase in phospho-JNK was clear at 3 h after D-Galn injection (Figs. 1 and 2A), when the oxidative stress had significantly increased as evidenced by decreased liver vitamin C, while liver necrosis was not evident based on plasma GOT and GPT levels (Table 1).

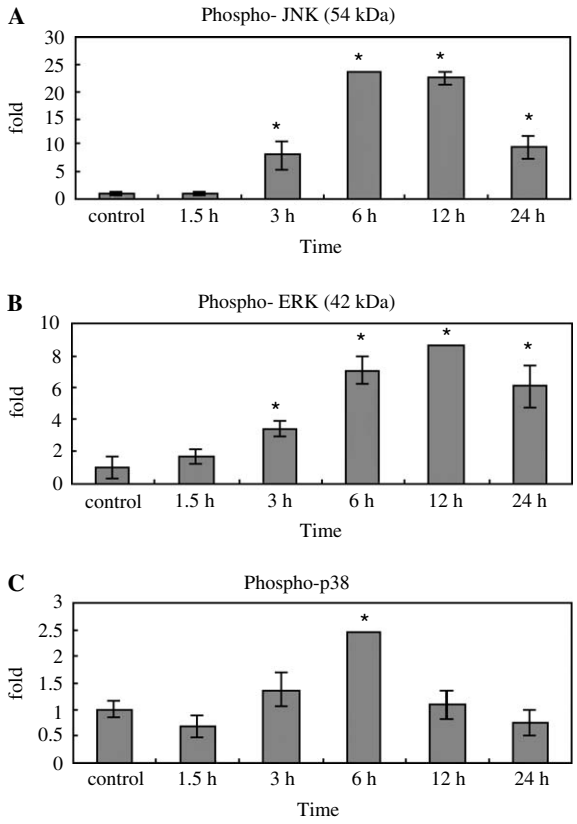

Figure 2. Densitometry of phospho-MAPK in the livers of rats 1.5, 3, 6, 12, and 24 h after D-galactosamine injections and control rats. D-Galactosamine (1 g/kg body weight) was intraperitoneally administered to rats. After 1.5, 3, 6, 12, and 24 h, protein concentrations of phosphorylated MAPK (described as phospho-ERK, phospho-p38, and phospho-JNK) in the livers were determined as described in the text. Three hours after the administration of saline, determinations were made for control rats. Values are means ± SE for four rats and asterisks indicate significant differences from the corresponding control group (ANOVA Fisher's protected least significant difference test (PLSD), $*P < 0.05$).

In a series of studies, we demonstrated that the tissue vitamin C concentration was the most sensitive indicator of oxidative stress[3] caused by carbon tetrachloride,[15] thioacetamide,[16] and D-Galn.[2] The liver vitamin C concentration decreased before extensive radical reactions leading to the accumulation of lipid hydroperoxides took place.[2] These results indicated that oxidative stress and the activation of JNK took place almost simultaneously, resulting in subsequent widespread cell death indicated by high levels of GOT and GPT (Table 1).

Along with JNK, p38 MAPK has been shown to have a similar activation profile[17,18] toward stresses such as ischemia–reperfusion,[11] growth factor withdrawal,[19] hypoosmotic conditions,[20] TGF-β1,[10] thioacetamide,[12] and 4-hydroxy-2-nonenal,[21] the end product of lipid peroxidation. This similarity may be ascribed to apoptosis signal-regulating kinase (ASK1), a common phosphorylation enzyme.[12,22] On the other hand, JNK and p38 MAPK were independently regulated under carbon tetrachloride intoxication[14] and repeated fasting stress.[23]

This study demonstrated that phospho-JNK increased at earlier phase than phospho-p38 MAPK after administration of D-Galn. This observation suggested that JNK and p38 MAPK were independently regulated as reported for carbon tetrachloride intoxication[14] and repeated fasting stress.[23] This result may be explained on the grounds that carbon tetrachloride shared a radical reaction as a common mechanism of liver cell death[15] with D-Galn.[2]

TGF-β1[10] and hypoosmotic stress[20] activated p38 MAPK, JNK, and ERK in hepatocytes. Although it is still controversial, ERK has been reported to be cytoprotective against apoptosis triggered by oxidative stress,[13,24,25] tumor necrosis factor α,[26,27] growth factor deprivation,[19] and by proapoptotic drugs,[28] in contrast to JNK and p38 MAPK. In addition, hepatocyte resistance to oxidative stress depended on protein kinase C and ERK-mediated downregulation of JNK signaling.[13] In the present study, phospho-ERK2 was significantly increased at 3 h and clearly elevated 6–24 h after the D-Galn injection (Figs. 1 and 2B). However, extensive apoptosis and necrosis took place 12 h after D-Galn administration. These findings imply that the activation of ERK took place, and that the strong apoptotic and necrotic signal induced by a high dose of D-Galn could block cell responses to growth and survival factors acting through the ERK pathway.

In this study, oxidative stress causing the decrease in vitamin C and the phosphorylation of JNK and ERK occurred almost simultaneously. Therefore, it remains to be clarified whether oxidative stress was a cause or/and a result of the activation of JNK and ERK, causing and preventing cell death, respectively. If oxidative stress were a cause of the phosphorylation of these MAPKs, their activation followed oxidative stress very rapidly. This result indicated closer relationship between oxidative stress and MAPKs than that formerly assumed.

In conclusion, administration of D-Galn rapidly caused oxidative stress and the activation of MAPKs such as JNK and ERK, followed by the activation of p38 MAPK.

Acknowledgment

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6. *Animal experiments*: Guidelines from the Prime Minister's Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals were followed. Male rats (SLC: Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). The animals were housed in a room at 24 ± 2 °C, with a 12 h/12 h light–dark cycle. Animals were fed commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum. Eight-week-old rats were intraperitoneally administered D-Galn (1 g/kg body weight). The control rats received saline and 3 h after treatment the liver and plasma were taken.
7. *Analytical methods*: Rats were anesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. Blood was centrifuged at 9000*g* for 5 min at 4 °C to separate plasma. After perfusion of ice-cooled saline through the portal vein, the organs were removed. The activity of plasma glutamate-oxaloacetate transaminase (GOT: EC 2.6.1.1) and glutamate-pyruvate transaminase (GPT: EC 2.6.1.2) was determined using diagnostic kits (GOT and GPT-UV Test Wako, Wako Pure Chemicals Co., Osaka) and expressed as Karmen Units. The excised tissue was homogenized in five volumes of phosphate-buffered saline (10 mM, pH 7.4) under ice cooling for vitamin C analysis. All determinations were made in duplicate experiments with four to six animals in each group. The determination of vitamin C was made according to a specific and sensitive method[29,30] involving chemical derivatization and HPLC. Data are expressed as means ± SD and analyzed using StatView software (Abacus Concepts, Berkeley, CA, USA). Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD) generated by this program.
8. *Western blot analysis of p38 MAPK, ERK, and JNK*: Liver tissue was removed and frozen at −83 °C until use. Homogenization was done basically as reported.[11] The extraction buffer contained 10 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5 mM EDTA, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM of sodium orthovanadate, 1 μg/mL of pepstatin A, 2 μg/mL of leupeptin, and 1 mM of phenylmethylsulfonyl fluoride (PMSF). Samples were homogenized in five volumes of the extraction buffer on ice. All debris and nuclei were removed by centrifugation at 9000*g* at 4 °C for 10 min, and the supernatant obtained was used for Western blot analysis. Protein concentrations were determined according to the method of Lowry et al.[31] using BSA as the standard. One hundred micrograms of protein was electrophoresed on a 10% SDS–PAGE gel and transferred to BioTrace NT membranes (Pall Gelman Laboratory, Ann Arbor, MI, USA). Detection of phosphorylated p38 MAPK, JNK, and ERK was done according to the instruction manual provided by Cell Signaling Technology Inc. (Beverly, MA, USA) using a PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) Antibody Kit, a PhosphoPlus SAPK/JNK (Thr183/Tyr185) Antibody Kit, and a PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) Antibody Kit, respectively. These kits were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Chemiluminescence was recorded with a cooled CCD camera system (Type AE-6972, ATTO Co. Ltd, Tokyo, Japan) and analyzed with ATTO Densitograph Software Library, CS Analyzer Ver2.0).
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