Analysis of In Vivo Nuclear Factor-κB Activation during Liver Inflammation in Mice: Prevention by Catalase Delivery[S]

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ABSTRACT

Nuclear factor-κB (NF-κB) is a transcription factor that plays crucial roles in inflammation, immunity, cell proliferation, and apoptosis. Until now, there have been few studies of NF-κB activation in whole animals because of experimental difficulties. Here, we show that mice receiving a simple injection of plasmid vectors can be used to examine NF-κB activation in the liver. Two plasmid vectors, pNF-κB-Luc (firefly *luciferase* gene) and pRL-SV40 (*Renilla reniformis luciferase* gene), were injected into the tail vein of mice by the hydrodynamics-based procedure, an established method of gene transfer to mouse liver. Then, the ratio of the firefly and *R. reniformis* luciferase activities (F/R) was used as an indicator of the NF-κB activity in the liver. Injection of thioacetamide or lipopolysaccharide plus D-galactosamine increased the F/R ratio in the liver, and this was significantly ($P < 0.001$) inhibited by an intravenous injection of catalase derivatives targeting liver nonparenchymal cells. Imaging the firefly luciferase expression in live mice clearly demonstrated that the catalase derivatives efficiently prevented the NF-κB-mediated expression of the firefly *luciferase* gene. Plasma transaminases and the survival rate of mice supported the findings obtained by the luminescence-based analyses. Thus, this method, which requires no genetic recombination techniques, is highly sensitive to the activation of NF-κB and allows us to continuously examine the activation in live animals. In conclusion, this novel, simple, and sensitive method can be used not only for analyzing the NF-κB activation in the organ under different inflammatory conditions but also for screening drug candidates for the prevention of liver inflammation.

Liver inflammation is a highly important event occurring in various conditions including ischemia/reperfusion injury, viral infection, drug-induced hepatic injury, and septic shock (Loguercio and Federico, 2003). The inflammation is normally associated with the induction of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ. Active transcription of these molecules leads to inflammatory damage to organs, in which activation of transcription factors takes place. Nuclear factor κB (NF-κB) is a transcription factor that plays crucial roles in inflammation,

immunity, cell proliferation, and apoptosis (Viatour et al., 2005). Activation of NF-κB mainly occurs via IκB kinase-mediated phosphorylation of inhibitory molecules, including IκBα. Expression of NF-κB-inducible genes also requires phosphorylation of NF-κB proteins, such as p65, by a variety of kinases in response to distinct stimuli. Because deregulation of NF-κB and IκB phosphorylation is a hallmark of long-term inflammatory diseases and cancer, newly designed drugs targeting these constitutively activated-signaling pathways are promising therapeutic agents. However, to date, NF-κB activation has not been properly investigated in whole animals because of experimental difficulties. Although antibodies and immunofluorescence assays of NF-κB can be used in mechanistic studies of specific compounds to detect NF-κB activation (van den Berg et al., 2001; Shen et al., 2002), these methods are cumbersome, complicated, and expensive. Therefore, a simple and reliable method to detect NF-κB activation is useful for screening drug candidates. Here, we report that mice receiving a simple injection of

ABBREVIATIONS: TNF, tumor necrosis factor; Gal-catalase, galactosylated catalase; Man-catalase, mannosylated catalase; PEG-catalase, polyethyleneglycol-conjugated catalase; Suc-catalase, succinylated catalase; TA, thioacetamide; LPS, lipopolysaccharide; GalN, D-galactosamine; IL, interleukin; ROS, reactive oxygen species; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; F/R, ratio of the firefly and *Renilla reniformis* luciferase activities; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SV40, simian virus 40.

plasmid vectors can be used as a model to examine NF-κB activity in the liver. Liver inflammation through NF-κB activation is induced by intraperitoneal injection of thioacetamide (TA) or lipopolysaccharide (LPS) plus D-galactosamine (GalN) in mice, and the inhibitory effect of chemically modified catalase derivatives targeting liver nonparenchymal cells on the TA- or LPS/GalN-induced inflammation is demonstrated.

Materials and Methods

Animals. Male C57/BL6 (6-week-old) and BALB/c (5-week-old) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University. BALB/c mice were used for the imaging of luciferase expression to avoid light extinction, and C57/BL6 mice were used for the other experiments.

Chemicals. TA, LPS (from *Escherichia coli* 0111:B4), GalN, and bovine liver catalase (C-100, 40,000 U/mg) were purchased from Sigma Chemical (St. Louis, MO). D-Luciferin was purchased from Promega (Madison, WI). Galactosylated (Gal-), mannosylated (Man-), polyethyleneglycol-conjugated (PEG-), or succinylated (Suc-) catalase was synthesized, and its enzymatic activity was measured as reported previously (Yabe et al., 1999a). All other chemicals were of the highest grade commercially available.

Plasmid DNA. pNF-κB-Luc encoding firefly *luciferase* gene driven by a basic promoter element plus five tandem copies of NF-κB binding elements [(TGGGGACTTTCCGC)$_5$] was purchased from Stratagene (La Jolla, CA). pRL-SV40 encoding the *Renilla reniformis luciferase* gene under the control of SV40 promoter, which has no NF-κB binding sequences, was purchased from Promega (Madison, WI). Plasmid DNA was injected into the tail vein of mice by the hydrodynamics-based procedure (Liu et al., 1999; Zhang et al., 1999; Kobayashi et al., 2001, 2002, 2004), which is an established method for in vivo gene transfer to the liver. According to previous reports, plasmid DNA dissolved in 1.5 ml of saline was injected into the tail vein within 5 s. Based on the sensitivity of the assays, the doses of plasmid DNA were set at 5 μg/mouse for pNF-κB-Luc for the in vivo imaging study and 0.1 μg/mouse each for pNFκB-Luc and pRL-SV40 for quantitative analysis.

Fulminant Liver Injury Model. TA (1000 mg/kg of body weight) or LPS (1 μg/body) plus GalN (10 mg/body) were injected intraperitoneally into mice 24 h after the injection of plasmid DNA to induce a fulminant liver injury. The dose of TA or LPS/GalN was determined according to previous reports (Hwang et al., 2003; Okuyama et al., 2003). Saline (untreated, control group) or any catalase derivative was injected in the tail vein at a dose of 5000 catalase U/mouse just before TA or LPS/GalN injection. The dose of catalase was determined according to our previous studies, in which 100 to 10,000 units of catalase derivatives were administered in various experimental settings (Yabe et al., 1999a,b, 2001; Hyoudou et al., 2004; Ma et al., 2006). These studies and ones with other chemically modified proteins (Nishikawa et al., 1995; Opanasopit et al., 2001; Yamasaki et al., 2002) demonstrated that the delivery efficiency of chemically modified proteins will decrease with increasing dose. Therefore, any dose greater than the dose used (5000 U/mouse, which is equivalent to approximately 5 mg/kg of body weight) was not tested in the present study. In addition, this dose falls in the range of standard doses (<10 mg/kg of body weight) of protein drugs. At 24 h after TA or LPS/GalN injection, the luciferase activities in the liver were measured as described below. The survival of mice was also examined in a different set of mice treated in the same manner except for the hydrodynamic delivery of pDNA.

Luciferase Assay. At 24 h after TA or LPS/GalN injection, the liver was excised, homogenized, and centrifuged as reported previously (Hyoudou et al., 2004). Then, 10 μl of the supernatant was mixed with 100 μl of firefly luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) to detect firefly luciferase activity or 100 μl *R. reniformis* luciferase assay buffer (Renilla Luciferase Assay System; Promega) to detect *R. reniformis* luciferase activity. The light produced was immediately measured using a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany). After subtraction of the background activity for the liver homogenate without injection, the ratio of the activity of the firefly luciferase to the *R. reniformis* luciferase was calculated to correct for differences in transfection efficiency among mice.

Luciferase Imaging. To avoid light extinction, white male BALB/c mice were used. At 24 h after TA or LPS/GalN injection, mice were injected intraperitoneally with 2 mg of D-luciferin, anesthetized with pentobarbital in phosphate-buffered saline, and then placed in a NightOwl LB 981 Molecular Light Imager (Berthold Technologies). Imaging was then performed in a two-step process using WinLight32 software. First, a black-and-white photographic image was acquired using a 15-ms exposure. Next, the luminescent image was acquired using a 5-min photon integration period with background subtraction. The luminescent image was processed by the software to color the luminescence intensity and then overlaid onto the photographic image. The parameters in the WinLight32 software used to obtain luminescent images were as follows: color threshold, 500 to 5000; color scheme, linear.

Determination of Plasma Transaminase Activities. At 24 h after TA or LPS/GalN injection, blood was collected from the vena cava using a heparinized syringe, and plasma was obtained by centrifugation. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the plasma were determined by commercially available test reagents (Transaminase test Wako; Wako, Osaka, Japan). Control values were determined using the blood obtained from age-matched, untreated mice.

Electrophoretic Mobility Shift Assay. At 12 h after TA or LPS/GalN injection, the liver was excised, and nuclear protein was extracted with a CelLytic Nuclear Extraction Kit (Sigma) according to the manufacturer's instructions. Then, the nuclear protein was adjusted to 5 μg/μl with water, and electrophoretic mobility shift assay (EMSA) was performed with EMSA Gel-Shift Kits (Panomics, Redwood City, CA), which contains consensus NF-κB binding oligonucleotide (AGTTGAGGGGACTTTCCCAGGC). Nuclear extracts from HeLa cells containing NF-κB protein were mixed with the labeled and unlabeled probe to obtain the positive and negative controls, respectively, and these were also used in the assay. Electrophoresis was performed with 7.5% polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). NF-κB-derived luminescent bands were detected by a cooled charge-coupled device camera (Light Capture AE-6962; Atto Corporation, Tokyo, Japan) according to the manufacturer's instructions. A quantitative densitometry analysis was performed by CS analyzer (Atto Corporation).

Statistical Analysis. Differences were statistically evaluated by one-way analysis of variance followed by the Student-Neumann-Keuls multiple comparison test and Kaplan-Meier analysis with a Wilcoxon test to determine survival, and the level of statistical significance was $P < 0.05$.

Results

Expression of Firefly and *R. reniformis* Luciferases After In Vivo Gene Transfer. The firefly luciferase and *R. reniformis* luciferase activities peaked at 8 h after injection and then decreased with time, but both luciferase activities were high enough to be detected even 72 h after injection.

Figure 1A shows the time course of the F/R ratio in the liver of mice that were injected with pNF-κB-Luc and pRL-SV40. The F/R ratio decreased with time from 3 to 24 h after pDNA injection and then remained constant after 24 h.

Then, we investigated the effects of TA or LPS/GalN on the firefly luciferase activity (Fig. 1B). An intraperitoneal injection of TA or LPS/GalN at 24 h after pDNA injection significantly ($P < 0.05$) increased the F/R ratio from 0.14 ± 0.03 (the saline-treated group) to 0.88 ± 0.24 and 0.52 ± 0.19 in the TA-treated and the LPS/GalN-treated groups, respectively, at 24 h after TA or LPS/GalN injection. These results clearly demonstrated that NF-κB is activated by TA or LPS/GalN. The *R. reniformis* luciferase activity in the TA-treated or the LPS/GalN-treated mice was not significantly different from that of saline-treated mice (data not shown).

Luminescence-Based Analysis of Thioacetamide-Induced NF-κB Activation in Mouse Liver. Because ROS activates NF-κB (Piette et al., 1997), we examined whether scavenging of ROS by catalase derivatives suppresses the expression of firefly luciferase gene in the present model. As shown in Fig. 2A, a large amount of firefly luciferase-derived luminescence was detected in the liver of the saline-treated live mice. The luminescence intensity was converted to a heat map image by WinLight32 software. An intravenous injection of native catalase, PEG-, or Gal-catalase (5000 U/mouse)

suppressed the expression to a degree. However, Man- or Suc-catalase, both of which are delivered to liver nonparenchymal cells (Yabe et al., 1999a), markedly reduced the luminescence in the liver. A quantitative luciferase assay gave results similar to those of the imaging study (Fig. 2B). Man- or Suc-catalase significantly ($P < 0.001$) reduced the F/R ratio in the liver, clearly demonstrating that the catalase derivatives targeting liver nonparenchymal cells efficiently prevent NF-κB activation.

Effect of Catalase Derivatives on Thioacetamide-Induced Liver Injury. The hepatic enzymes, AST and ALT, are frequently used as indicators of liver damage. It has been reported that TA causes lethal liver damage with high serum AST and ALT activities (Diez-Fernandez et al., 1993). In the present model, a marked increase in AST and ALT levels was
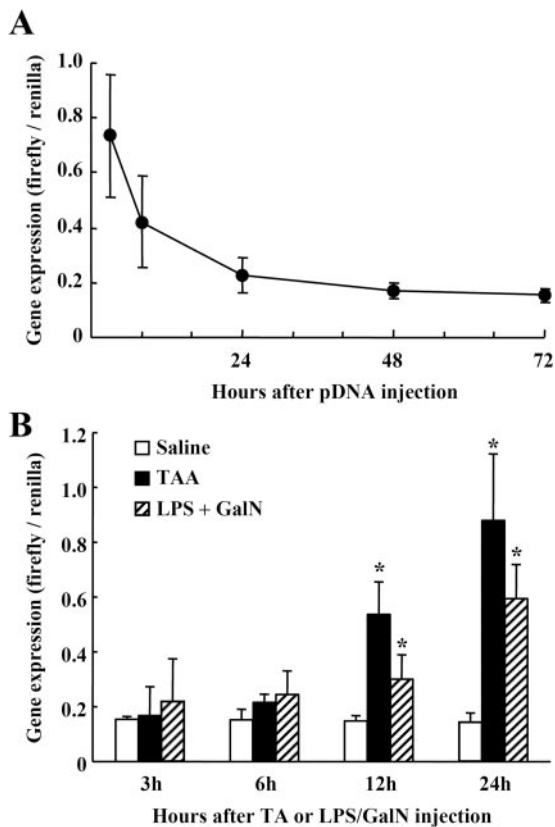

Fig. 1. The ratio of firefly and *R. reniformis* luciferases (F/R ratio) in the liver. A, mice were killed at 3, 8, 24, 48, or 72 h after pDNA injection, and the luciferase activity in the liver was assayed. Results are expressed as the mean ± S.D. of at least three mice. B, effect of TA or LPS/GalN on the F/R ratio. TA or LPS/GalN was intraperitoneally injected 24 h after pDNA injection, and the luciferase activity in the liver was assayed 3, 6, 12, or 24 h after TA or LPS/GalN injection. Results are expressed as the mean ± SD of at least three mice. ∗, a statistically significant difference compared with the saline-treated group ($P < 0.05$).
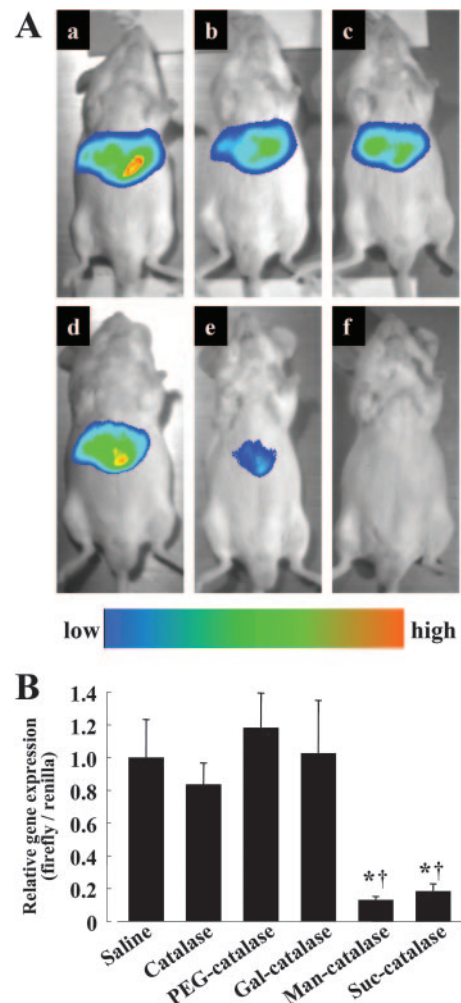

Fig. 2. Effect of catalase derivatives on the activation of NF-κB. TA was intraperitoneally injected 24 h after pDNA injection. Saline (vehicle) or any catalase derivative (5000 units/mouse) was intravenously injected just before the TA injection. A, visualization of firefly *luciferase* gene expression in live mice 24 h after TA injection. a, saline; b, catalase; c, PEG-catalase; d, Gal-catalase; e, Man-catalase; f, Suc-catalase. The luminescent signals were color-coded based on the color scale below the images. B, mice were killed 24 h after TA injection, and the luciferase activities in the liver were assayed. Relative gene expression was indicated as an *x*-fold increase compared with the saline-treatment group. Results are expressed as the mean ± SD of four mice. ∗, a statistically significant difference compared with the saline-treated group ($P < 0.001$); †, a statistically significant difference compared with the catalase-treated group ($P < 0.001$).

detected after TA administration to mice (Fig. 3, A and B). Intravenous injection of any catalase derivative (5000 U/mouse) inhibited the increase in the plasma AST and ALT levels, with significant ($P < 0.05$) inhibition by Man- or Suc-catalase.

Figure 4 shows the survival of mice receiving an intraperitoneal injection of TA. Man- or Suc-catalase significantly increased the survival period compared with the saline-, catalase-, PEG-catalase-, or Gal-catalase-treated group ($P < 0.01$ for the saline-treated group, $P < 0.05$ for the catalase-, PEG-catalase-, or Gal-catalase-treated group).

NF-κB Activation Induced by Lipopolysaccharide Plus D-Galactosamine and Its Prevention by Catalase Derivatives. The above results demonstrated that the proposed luminescence-based analytical method is effective in evaluating TA-induced fulminant liver injury and its prevention by the catalase derivatives, Man-catalase and Suc-cata-

lase. To examine the versatility and reliability of this analytical method when applied to other disease models, the effect of catalase derivatives on LPS/GalN-induced fulminant liver injury was also investigated. As shown by the quantitative luciferase assay in Fig. 1B, a large amount of firefly luciferase-derived luminescence was detected in the saline-treated mice after injection of LPS/GalN (Fig. 5A). Treatment with catalase, PEG-, or Gal-catalase reduced the luminescence in live mice to a degree, whereas Man- or Suc-catalase markedly inhibited the expression of the firefly *luciferase* gene. A quantitative analysis supported these results (Fig. 5B); Man- or Suc-catalase significantly ($P < 0.05$) reduced the F/R ratio in the liver of LPS/GalN-treated mice. Plasma ALT and AST levels that were markedly increased by the LPS/GalN treatment were significantly ($P < 0.05$) inhibited by Man- or Suc-catalase (Fig. 6, A and B). The survival of LPS/GalN-treated mice was also significantly ($P < 0.01$ for the saline-, catalase-, PEG-catalase-, or Gal-catalase-treated group) increased by Man- or Suc-catalase (Fig. 7).

EMSA of Liver Nuclear Extract. To confirm the results obtained using pDNA, we performed an EMSA analysis of nuclear NF-κB in mouse liver (Fig. 8). The NF-κB activity was markedly increased by TA or LPS/GalN. This activation was effectively inhibited by Man-catalase. These results strongly support that hypothesis that the data obtained by the newly developed method using pDNA reflect changes in NF-κB activity in the liver during inflammation.

Discussion

NF-κB is a ubiquitous transcription factor that is involved in many inflammatory processes, and the importance of NF-κB activation has been widely discussed in relation to liver injury. One of the most popular approaches to analyzing NF-κB activation is EMSA. A standard EMSA typically relies upon ³²P-end-labeling of nucleic acids to detect the DNA component of the protein-DNA complexes on polyacrylamide gels. Although isotopic labeling provides high-detection sensitivity, the use of radioisotopes is subject to regulatory procedures, disposal limitations, and the short half-life of the
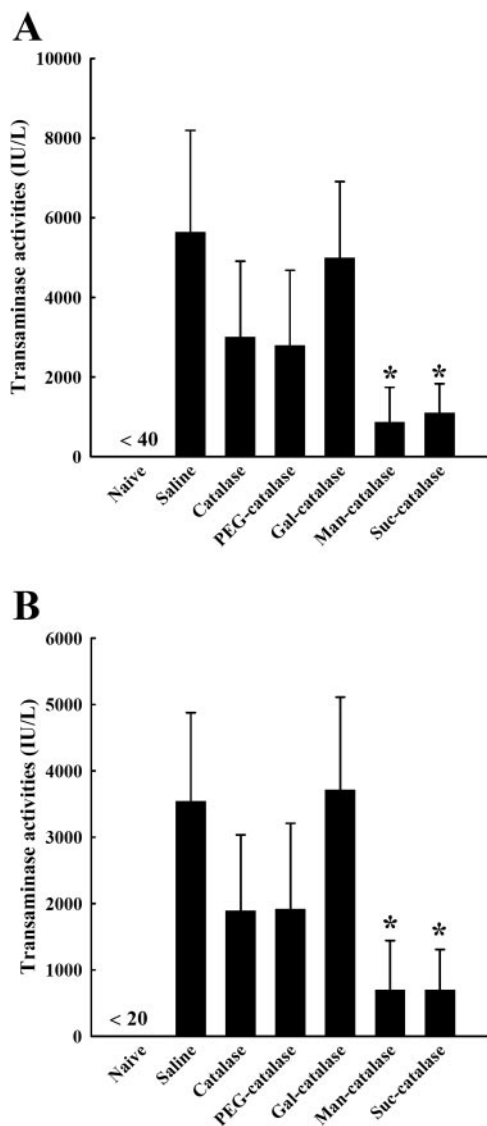

Fig. 3. Effect of catalase derivatives on plasma transaminase levels. Saline (vehicle) or any catalase derivative (5000 U/mouse) was injected into the tail vein of mice just before the intraperitoneal TA injection. Results are expressed as the mean ± SD of four mice. *, a statistically significant difference compared with the saline-treated group ($P < 0.05$). A, aspartate aminotransferase (AST). B, alanine aminotransferase (ALT).
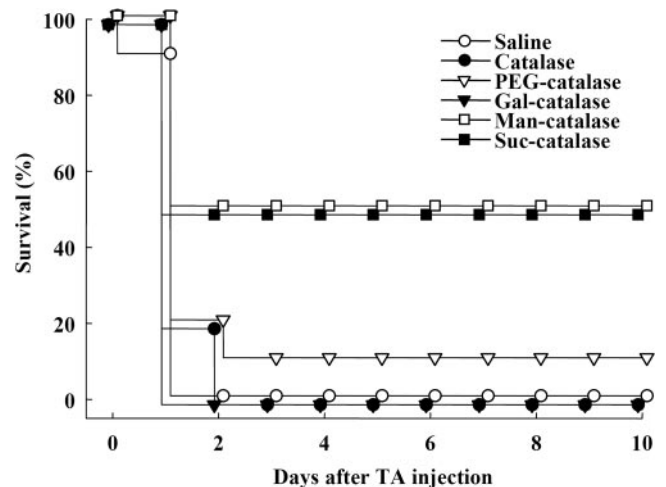

Fig. 4. Survival rate of mice receiving an intraperitoneal injection of TA. Saline (vehicle) or any catalase derivative (5000 U/mouse) was injected into the tail vein. The survival of the Suc- or Man-catalase treated group was significantly greater than that of the saline- ($P < 0.01$) or catalase-treated ($P < 0.05$) group ($n = 10$).

label, and requiring a long incubation with an autoradiographic film. In marked contrast, the luminescence-based technology developed in the present study provides a simple, sensitive, and quantitative method for detecting the real-time transcriptional activity of NF-κB in live animals. This luminescence-based method consists basically of in vivo gene transfer to mouse liver using two plasmid vectors: one is pNF-κB-Luc, which encodes firefly *luciferase* gene driven by a basic promoter element plus NF-κB binding elements, and the other is pRL-SV40, which encodes *R. reniformis luciferase* gene under the control of an NF-κB-insensitive SV40 promoter. pNF-κB-Luc is designed especially for monitoring activation of the NF-κB signal transduction pathway, which contains five tandem copies of the NF-κB enhancer element. We confirmed the response of firefly *luciferase* expression in
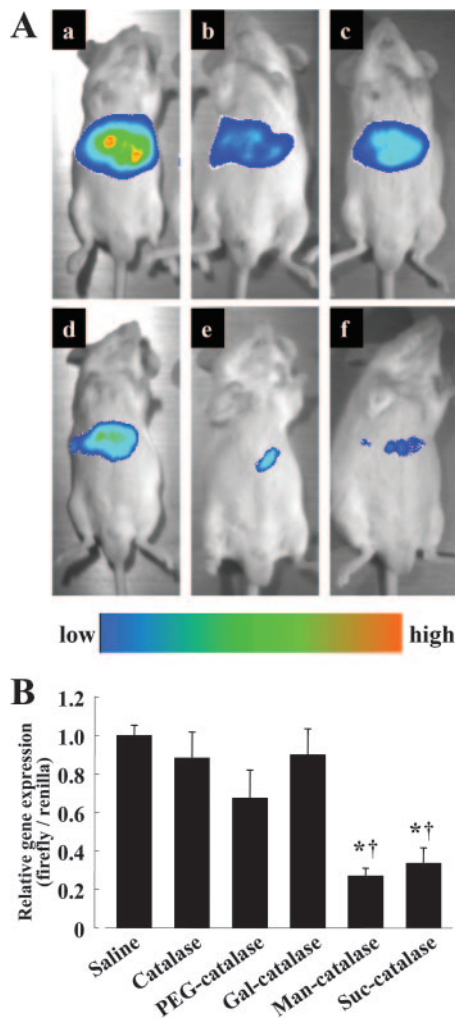
mouse melanoma B16-BL6 cells permanently transfected with pNF-κB-Luc when activated by standard stimuli, such as hydrogen peroxide (Supplemental Figure) or TNF-α (Y. Kobayashi, M. Nishikawa, K. Hyoudou, F. Yamashita, and M. Hashida, unpublished data). We also confirmed that the results obtained using pDNA correspond to those obtained by the EMSA of NF-κB in mouse liver. Therefore, this method is specific for the real transcriptional activity of NF-κB. Moreover, this approach can be applied to analyzing the changes in other transcription factors that are associated with liver inflammation, such as activator protein-1, by simply replacing pNF-κB-Luc with such transcription factor specific vectors. NF-κB consists of homodimers or heterodimers through the combination of the subunits p65 (Rel-A), p50 (NF-κB$_1$, p105), p52 (NF-κB$_2$, p100), c-Rel, or Rel-B. It has already been reported that TA (Fernandez-Martinez et al., 2004) and LPS (Romics et al., 2004) induces nuclear localization of
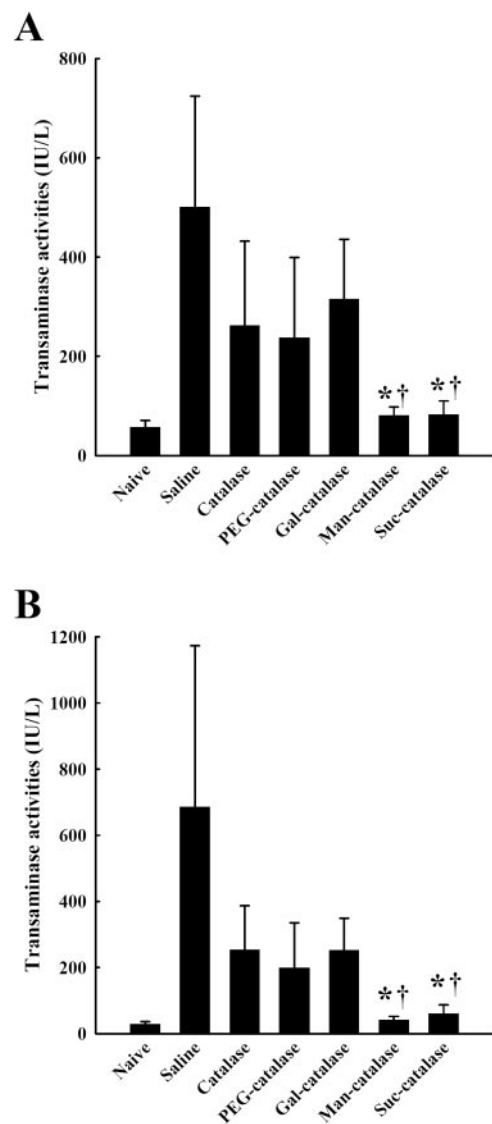

Fig. 5. Effect of catalase derivatives on the activation of NF-κB. LPS/GalN was intraperitoneally injected 24 h after pDNA injection. Saline (vehicle) or any catalase derivative (5000 U/mouse) was intravenously injected just before the LPS/GalN injection. A, visualization of firefly *luciferase* gene expression in live mice 24 h after LPS/GalN injection. a, saline; b, catalase; c, PEG-catalase; d, Gal-catalase; e, Man-catalase; f, Suc-catalase. The luminescent signals were color-coded based on the color scale below the images. B, mice were killed 24 h after LPS/GalN injection, and the luciferase activities in the liver were assayed. Relative gene expression was indicated as an *x*-fold increase compared with the saline-treatment group. Results are expressed as the mean ± SD of four mice. *, a significant difference compared with the saline-treated group ($P < 0.01$); †, a statistically significant difference compared with the catalase-treated group ($P < 0.01$).

Fig. 6. Effect of catalase derivatives on plasma transaminase levels. Saline (vehicle) or any catalase derivative (5000 U/mouse) was injected into the tail vein of mice just before the intraperitoneal LPS/GalN injection. Results are expressed as the mean ± SD of five mice. *, a significant difference compared with the saline-treated group ($P < 0.05$); †, a statistically significant difference compared with the catalase-treated group ($P < 0.05$). A, AST. B, ALT.

p65/p50 and p50/p50 complexes. Therefore, although the two bands detected in the EMSA were not identified, they would represent p65/p50 and p50/p50 dimers.

In the present study, the reporter genes were introduced into mouse liver by intravenous injection of pDNA using the hydrodynamics-based procedure, an established gene transfer method to the liver (Liu et al., 1999). This method gives very high transgene expression with naked pDNA, although a transient increase in liver enzymes within 1 day of the hydrodynamics-based gene transfer has been reported (Kobayashi et al., 2004). As shown in Fig. 1A, the F/R ratio was almost constant 1 day after pDNA injection, suggesting that the hydrodynamics-based procedure itself induces very little toxicity at 1 day after injection or later. The mechanism for the hydrodynamics-based gene transfer would involve the direct cytosolic delivery of pDNA through the cell membrane as a result of transiently increased permeability (Kobayashi et al., 2001, 2004). Moreover, hepatic uptake and gene expression mechanisms after intravenous administration of pDNA by conventional and hydrodynamics-based procedures have been reported previously (Kobayashi et al., 2001). In the present study, we tried to minimize the dose of pDNA. For the quantitative luciferase assay, 0.1 μg of pDNA was sufficient for the analysis. On the other hand, at least 5 μg of pDNA was needed for the imaging. The sensitivity of the assay systems explains the difference in the doses. Within this dose range, the level of transgene expression after the hydrodynamics-based procedure is reported to be proportional to the dose (Kobayashi et al., 2004). White BALB/c mice were used for the in vivo imaging studies, because luciferin chemiluminescence can hardly be detected through the black color of C57BL/6 mice.

It has been reported that the administration of TA activates NF-κB followed by the generation of ROS in the liver (Lu et al., 1999), and it was also reported that LPS treatment increases the generation of ROS in Kupffer cells (Uchikura et al., 2004) and the NF-κB binding activity in hepatocytes (Freedman et al., 1992). Reactive oxygen species contribute to the pathogenesis of a variety of liver diseases, such as acetaminophen overdose, hemochromatosis, alcoholic liver injury, toxin exposure, and viral hepatitis (Slater, 1984). Proinflammatory cytokines, especially TNF-α, interleukin (IL)-1, IL-6, and IL-18, are believed to play roles in the cellular injury associated with fulminant hepatitis. It has been reported that hydrogen peroxide is able to directly activate NF-κB, and a variety of antioxidants and antioxidant enzymes, such as catalase, down-regulate TNF-α-mediated NF-κB activation (Schreck et al., 1991; Anderson et al., 1994). TA is a thiono-sulfur-containing compound that is commonly used for inducing fulminant hepatic failure (Bruck et al., 2002) and liver cirrhosis (Kamath et al., 1999) in animal models. During the biotransformation of TA, both flavin-containing monooxygenase (Chieli and Malvaldi, 1984) and cytochrome P450 (Lee et al., 2003) reduce dioxygen to superoxide anion, which is then catalyzed to produce hydrogen peroxide. Therefore, biotransformation of TA precedes oxidative damage-associated liver injury. This injury has been implicated in the phenomena of glutathione depletion (Sanz et al., 2002), an increase in malondialdehyde (So et al., 2002), and the disappearance of tetraploid hepatocytes (Diez-Fernandez et al., 1993) in liver cells after TA administration. Nevertheless, the detailed biochemical mechanisms underlying this hepatotoxic process of TA remain largely unknown. Kupffer cells are reported to play an important role in TA-induced liver injury, because the depletion of
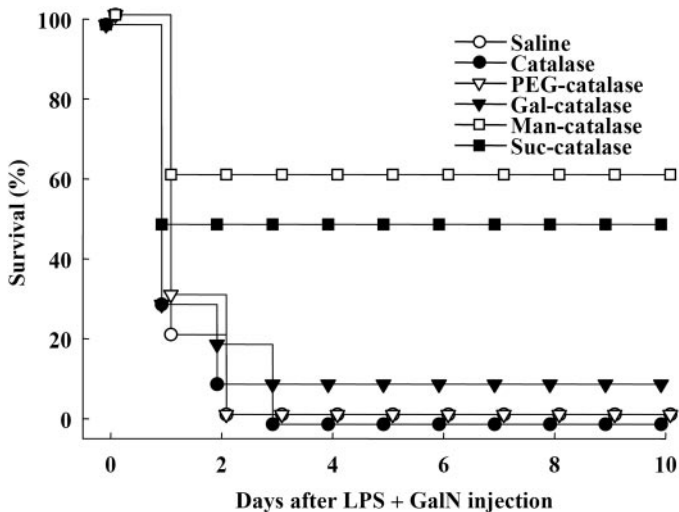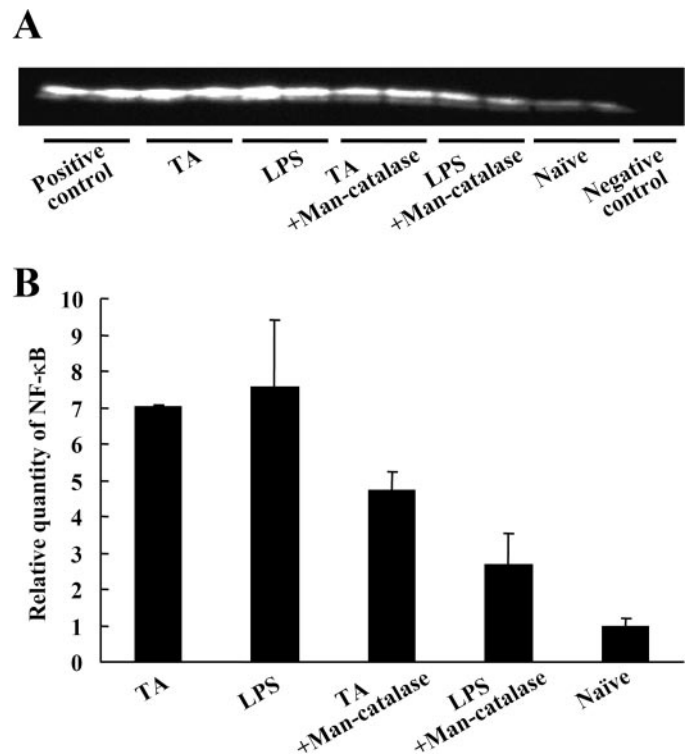

Fig. 7. Survival rate of mice receiving an intraperitoneal injection of LPS/GalN. Saline (vehicle) or any catalase derivative (5000 U/mouse) was injected into the tail vein. The survival of the Suc- or Man-catalase treated group was significantly ($P < 0.01$) greater than that of any other group ($n = 10$).

Fig. 8. EMSA analysis of NF-κB activity in nuclear extracts of mouse liver. Saline (vehicle) or any catalase derivative (5000 U/mouse) was injected into the tail vein of mice just before the intraperitoneal TA or LPS/GalN injection. A, activation of NF-κB binding to DNA as demonstrated by EMSA. Only the band for bound NF-κB is shown. The bands express the NF-κB activity in the liver of individual mice. Nuclear extracts from HeLa cells containing NF-κB protein were mixed with the labeled and unlabeled probe to obtain the positive and negative controls, respectively. B, quantitative densitometry evaluations of EMSA. Data are represented as an x-fold increase relative to the naive group. Results are expressed as the mean ± SD of two mice.

Kupffer cell function by gadolinium chloride attenuates TA-induced hepatotoxicity (Andres et al., 2003). In the present study, TA-induced NF-κB activation and liver injury was effectively inhibited by Man- or Suc-catalase, both of which are derivatives targeting Kupffer cells and liver endothelial cells through the recognition by mannose receptors or scavenger receptors, respectively. These results suggest that hydrogen peroxide is initially produced during metabolism of TA, and then, the peroxide generated distributes to all types of liver cells and activates NF-κB in receiving cells, followed by the production of inflammatory cytokines. In the LPS/GalN-induced fulminant hepatitis model, Man- or Suc-catalase also effectively inhibited NF-κB activation and liver injury. Because LPS is mainly recognized by the toll-like receptor 4 expressed on Kupffer cells (Su et al., 2000) and subsequently produce ROS (Uchikura et al., 2004) and inflammatory cytokines (Su et al., 2000), the delivery of catalase to the nonparenchymal cells is efficient. The cause of TA- or LPS/GalN-induced death is reported to be ROS-mediated liver dysfunction (Okuyama et al., 2003). In these models, mice will die within a couple of days, as observed in the present study, because of a rapid production of toxic ROS after treatment. If mice can tolerate the deadly assaults of ROS, they would survive for a relatively long period of time because little ROS are generated in the later periods.

Using various chemically modified catalase derivatives, we have shown the importance of the tissue distribution of such derivatives on their pharmacological activities (Yabe et al., 1999a,b, 2001; Nishikawa et al., 2004, 2005; Nishikawa and Hashida, 2006). In the present study, we demonstrated that Man-catalase and Suc-catalase were effective in inhibiting liver inflammation, whereas unmodified catalase or Gal-catalase, derivatives targeting liver parenchymal cells (hepatocytes), was not so effective. These results clearly demonstrate that the delivery of catalase to liver nonparenchymal cells, such as Kupffer and sinusoidal endothelial cells, is important for inhibition of the TA- or LPS/GalN-induced liver inflammation. These catalase derivatives, retaining high enzymatic activities (> 96%) after modification, were also effective in preventing ischemia/reperfusion hepatic injury in mice (Yabe et al., 1999b, 2001).

In conclusion, a quantitative method to analyze NF-κB activation in the liver has been successfully developed. Moreover, the method clearly demonstrates that catalase derivatives targeting liver nonparenchymal cells can effectively inhibit TA- or LPS/GalN-induced fulminant hepatic injury. This luminescence-based method requires no genetic recombination technique, is highly sensitive to the activation of NF-κB in the liver, and allows continuous monitoring of the activation in live animals. Because liver inflammation is a common pathological event, this novel, simple, and sensitive method can be used not only for analyzing the incidence of events in the organ under various inflammatory conditions but also for screening drug candidates for the prevention of the diseases involved in NF-κB activation, such as inflammation, oncogenesis, and tumor metastasis.

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