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Sensitivity of Metallothionein-Null Mice to LPS/D-Galactosamine-Induced Lethality

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Mice treated with lipopolysaccharide (LPS)/D-galactosamine (GalN) selectively develop hepatic failure. The acute-phase protein α_1-acid glycoprotein (AGP) has been demonstrated to protect mice from LPS/GalN-induced lethality. Metallothionein (MT), which is a low-molecular weight, cysteine-rich, metal-binding protein, is also induced in the acute-phase reaction. However, the specific function of MT in acute-phase response remain to be elucidated. We showed that MT-null mice were more sensitive to LPS/GalN-induced lethality than wild-type mice. The increase in vital mediator levels, TNF-α and NO were of similar levels in wild-type and MT-null mice. A remarkable increase in plasma platelet-activating factor levels was not observed in our experimental conditions. On the other hands, the mRNA level of AGP in the response to LPS/GalN was decreased in MT-null mice compared to wild-type mice. These results indicated that MT may have the potential to prevent LPS/GalN-induced lethality, at least through the attenuation of AGP induction.

Key Words: **metallothionein; lipopolysaccharide; D-galactosamine; α_1-acid glycoprotein.**

Bacterial lipopolysaccharide (LPS) is the primary pathogenic factor in gram-negative bacteria. Mice injected with a high dose of LPS suffer multiple organ failure, as characterized by circulatory response syndrome and septic shock. Following administration of LPS, the liver responds by markedly increasing the synthesis of a subset of serum proteins, known as the acute-phase proteins (1). Some of these acute-phase proteins have been reported to protect experimental animals from lethal endotoxemia or other inflammatory challenges (2–7). Metallothionein (MT), which is a low-molecular weight, cysteine-rich, metal-binding protein, is known to be induced in the acute-phase response (8). However, the specific functions of MT in acute-phase response remain to be elucidated.

MT-deficient (MT-null) mice were generated by homologous recombination of MT-I and -II genes (9, 10). These mice have been used to elucidate the roles of MT in the homeostasis of essential metals (11, 12), and detoxification of heavy metals (9, 10, 13), cisplatin (14), and paracetamol (15). Recently we suggested that MT modulates gene expression during development (16). In this study, we showed that MT-null mice are more sensitive to LPS/D-galactosamine (GalN)-induced lethality than wild-type mice. In addition, the mRNA level of α_1-acid glycoprotein (AGP) in the response to LPS/GalN was decreased in MT-null mice compared to wild-type mice. Our results suggest that MT may have the potential to up-regulate a protective protein, but not to modulate the levels of vital mediators.

MATERIALS AND METHODS

Reagents. LPS from *Escherichia coli* O26:B5 was obtained from Difco laboratories (Detroit, MI). Recombinant mouse tumor necrosis factor (TNF)-α was obtained from Pepro Tech, Inc. (Canton, MA). GalN and platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was purchased from Sigma Chemical Company (St. Louis, MO).

Animals. Male 8 to 12 week-old MT-null mice and their corresponding controls (129/Sv) were provided by Jackson Laboratory (Bar Harbor, ME) and maintained as a closed colony in our laboratory. All mice were housed under conditions of controlled temperature (23–24°C) and light (12 h light and 12 h dark). Food and tap water were provided *ad libitum*.

Animal experiments. The experiments were conducted in accordance with the institutional guidelines of Osaka University. Wild-type and MT-null mice were intraperitoneally administrated LPS (100 μg/kg) and GalN (700 mg/kg). The survival rate of mice over the next 24 h was recorded. Blood samples were taken under ether anesthesia to determine levels of TNF-α and nitric oxide (NO) metabolites. For plasma preparation, 450 μl blood was collected by a syringe containing 50 μl 0.1 M sodium citrate. Plasma was then obtained by centrifugation for 15 min at 16,000*g*.

Assay of TNF and NO metabolite serum levels. Serum TNF levels were determined by a L929 bioassay (17). Concentrations of nitrate

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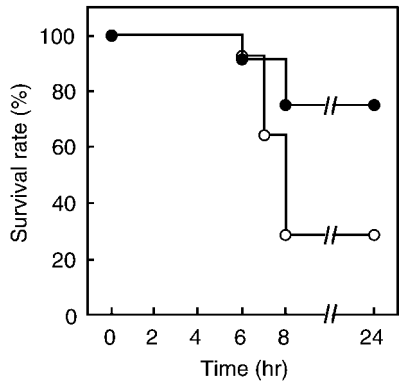

FIG. 1. Survival curve of wild-type (●, $n = 12$) and MT-null mice (○, $n = 14$) in response to intraperitoneal administration of LPS (100 μg/kg) plus GalN (700 mg/kg). Significant differences were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Survival rates in wild-type and MT-null mice were significantly different at $P < 0.05$.

and nitrite were measured to estimate NO production. Briefly, nitrate was enzymatically reduced to nitrite using nitrate reductase, and nitrite was then determined by a spectrophotometric method based on the Griess reaction (1% sulfanilamide/0.1% naphthylenediamine/2% phosphoric acid) (18).

Assay of PAF. Plasma PAF levels were determined by a platelet secretion assay using washed rabbit platelets. PAF in plasma was extracted by a reverse-phase octadecyl column (Amprep Minicolumn, Amersham International, Bucks, UK) (19). The PAF extract was then added to the washed rabbit platelet suspension to secrete serotonin (20), which was then reacted with OPT reagent (0.05% *O*-phthaldialdehyde/10% ethanol/8 M HCl) to form a fluorescent substance (Ex; 360 nm, Em; 475 nm).

Northern blot hybridization. Total RNAs isolated from wild-type and MT-null mice before and after the administration of LPS/GalN were resolved by electrophoresis in a 1.0% agarose/10% formaldehyde gel and transferred onto a nylon membrane (Amersham) in 20× SSC buffer. Northern blot hybridization was carried out with ^{32}P-labeled specific probes for AGP (21). The membrane was reprobed with a ^{32}P-labeled DNA probe for mouse 18S rRNA, which served as an internal control.

Statistial analysis. Significant differences in survival rate were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Significant differences in the other data were analyzed by ANOVA and Fisher's PLSD test. Differences between groups were considered significant at the $P < 0.05$ level.

RESULTS

Lethal Effect of LPS/GalN in MT-Null Mice

We investigated whether MT can resist to lethality induced by LPS/GalN. Figure 1 shows that MT-null mice exhibited high sensitivity to the lethal effects of LPS/GalN compared to wild-type mice. At 24 h after LPS/GalN administration, the survival rate was only 28.6% for MT-null mice compared with 75.0% for wild-type mice ($P < 0.05$).

Elevation of TNF and NO Metabolite Levels in Serum and PAF Levels in Plasma after LPS/GalN Administration

LPS/GalN administration resulted in the up-regulated production of various proinflammatory cytokines and inflammatory factors, including TNF-α (22), NO (23), and PAF (24), all of which play crucial roles in the lethal effects of LPS. The maximal levels of TNF-α and NO elevated in plasma following LPS/GalN administration (at 1 and 5 h, respectively) revealed no significant differences in TNF and NO metabolite levels between wild-type and MT-null mice (Figs. 2A and 2B). As demonstrated in Fig. 2C, the basal plasma PAF levels in MT-null mice were not significantly different from those of the wild-type mice (2.27 ± 0.68 μM vs 2.50 ± 0.55 μM). After LPS/GalN administration, plasma PAF levels were increased significantly in both wild-type and MT-null mice. Two hours after the administration of LPS/GalN, the PAF level in plasma of MT-null mice was approximately 1.5-fold higher than that in the wild-type mice (3.69 ± 0.12 μM vs 4.31 ± 0.56 μM, $P < 0.05$).

Messenger RNA Level of α_1-Acid Glycoprotein after LPS/GalN Administration

The mRNA levels of AGP were determined by Northern blot analysis (Fig. 3). AGP expression was induced by the administration of LPS/GalN in wild-type mice. However, the mRNA level of AGP in the response to LPS/GalN was decreased in MT-null mice compared to wild-type mice.

DISCUSSION

In this study, we showed that MT-null mice are more sensitive to LPS/GalN-induced lethality than wild-type mice. The levels of vital mediator, TNF, NO, and PAF, showed similar levels in wild-type and MT-null mice, whereas the mRNA levels of the protective protein, AGP, in the response to LPS/GalN was decreased in MT-null mice compared to wild-type mice. These results suggest that MT may have the potential to up-regulate a protective protein, but not to modulate the levels of vital mediators.

Some of the acute-phase proteins, such as AGP (5), α_1-antitrypsin (AT) (3, 6), C-reactive protein (4, 7), and serum amyloid A (2), have protected experimental animals from lethal endotoxemia or other inflammatory challenges. It is widely documented that acute-phase proteins are primarily involved in maintaining physiological homeostasis. In the LPS/GalN model, AGP and AT reportedly protect against LPS/GalN-induced lethality (5, 6). However, AT is not expressed in mice. AGP is the only acute-phase protein that protects mice from LPS/GalN-induced lethality. AGP is able to pre-

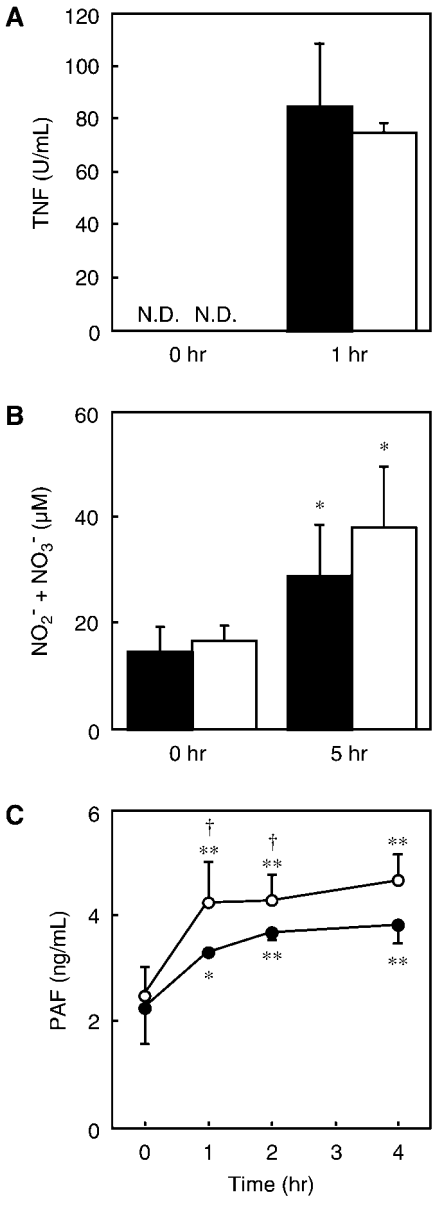

FIG. 2. Serum TNF (A) and NO metabolites (B) levels as well as plasma PAF levels (C) in wild-type (■, ●) and MT-null (□, ○) mice before and after administration of LPS plus GalN. Data represent means ± SD of $n = 3$–7 animals per group. Significantly different from control group (*$P < 0.01$; **$P < 0.001$), and significantly different from wild-type mice (†$P < 0.05$). N.D., not detectable.

vent LPS/GalN-induced apoptosis of hepatocytes (25), and has potent platelet aggregation-inhibitory activity (26). LPS/GalN-induced apoptosis of hepatocytes is an early and necessary step in the induction of lethality (22), and platelet aggregation and adhesion to the endothelium plays a critical role in the LPS/GalN model (27). These AGP activities are believed to be the source of its protective capacity. Our results show that the induction of AGP was decreased in MT-null mice compared with wild-type mice (Fig. 3). Furthermore, preliminary studies using primary cultured hepatocytes from wild-type and MT-null mice indicated that the mRNA levels of AGP in the response to interleukin-6, which mediates the induction of AGP, was decreased in hepatocytes from MT-null mice. Therefore, we thought that MT has the potential to up-regulate a protective protein in hepatocytes. The sensitization of MT-null mice may thus be caused by insufficient induction of AGP.

When discussing the potential mechanisms by which MT-null mice are sensitive to LPS/GalN-induced lethality, we primarily hypothesized that the sensitization was due to the increase in vital mediator levels, e.g., TNF-α (22), NO (23), and PAF (24). There is agreement among researchers that hepatic failure and death in LPS/GalN-administrated mice results from TNF-α toxicity. TNF-α formation in Kupffer cells is sensitive to the intracellular redox status because TNF-α formation is dependent on the redox-sensitive transcriptional factor, NF-κB (28). Furthermore, NO may play a role in LPS/GalN-induced lethality. The cytotoxic molecule NO is mainly synthesized by an inducible isoform of NO synthase (iNOS). Cytotoxicity usually correlates with NO produced by iNOS (29). The expression of iNOS is also regulated by NF-κB (30). Recent reports suggest that MT can modulate the activity of NF-κB (31, 32). However, serum TNF and NO levels in the MT-null mice in the present study were not significantly higher than those in the wild-type mice (Figs. 2A and 2B). The other vital mediator, PAF, is an endogenously produced phospholipid mediator that contributes to the pathogenesis of inflammation and tissue injury induced by LPS. PAF production from various cells such as neutrophils, macrophages, vascular endothelial cells, and platelets is known to be enhanced by LPS. However, a remarkable increase in plasma PAF levels was not observed in the wild-type or MT-null mice under our experimental conditions (Fig. 2C).

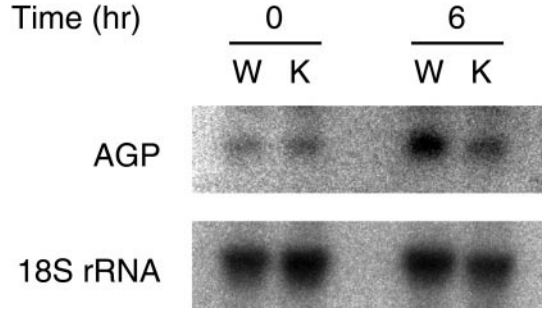

FIG. 3. Northern blot hybridization of AGP. Total RNAs were isolated from wild-type (W) and MT-null (K) mice before and 6 h after administration of LPS/GalN. A nucleotide 451–613 of the mouse AGP mRNA sequence reported by Lee *et al.* (24) and a nucleotide 165–203 of mouse 18S rRNA were used as mouse AGP and 18S rRNA probes, respectively. This experiment was done with total RNAs isolated from wild-type ($n = 3$) and MT-null mice ($n = 4$), and similar results were obtained. The results of one representative experiment are shown.

These data show that MT-null mice were more susceptible to LPS/GalN-induced lethality despite the slight elevation in TNF, NO, and PAF levels.

Several factors modulate lethality during endotoxemia. Many *in vivo* experiments demonstrated the importance of reactive oxygen species (ROS) in the pathophysiology (33). Since MT is thought to function as a free oxygen scavenger (34, 35), it may prevent LPS/GalN-induced lethality through this activity. Glutathione peroxidase (Gpx)-1 is the most abundant, cytosolic, and mitochondrial glutathione peroxidase. It was reported that Gpx1-null mice are more susceptible to LPS/GalN-induced lethality (36). Glutathione peroxidase has been thought to function in cellular antioxidant defense. Therefore, the free radical scavenging activity of MT also might be involved in the prevention of LPS/GalN-induced lethality.

In conclusion, the findings of the present study suggest that MT has the potential to up-regulate a protective protein during endotoxemia. MT levels are known to alter under varying conditions, e.g., upon exposure to heavy metals, deficiency of essential dietary metals, liver regeneration, and endotoxemia. However, the specific functions of MT in various conditions are not well understood. Many experiments demonstrated only that the functions of MT are the result of heavy metals detoxification (9, 10, 13) and homeostasis of essential metals (11, 12). The prevention of LPS/GalN-induced lethality by MT is interesting in that it is a novel function and underlines the importance of further elucidating MT function. Experiments investigating the mechanism of the insufficient induction of AGP by MT deficiency will be followed with great interest.

REFERENCES

1. Kushner, I. (1982) The phenomenon of the acute phase response. *Ann. N.Y. Acad. Sci.* **389,** 39–48.
2. Shainkin-Kestenbaum, R., Berlyne, G., Zimlichman, S., Sorin, H. R., Nyska, M., and Danon, A. (1991) Acute phase protein, serum amyloid A, inhibits IL-1- and TNF-induced fever and hypothalamic PGE_2 in mice. *Scand. J. Immunol.* **34,** 179–183.
3. Bucurenci, N., Blake, D. R., Chidwick, K., and Winyard, P. G. (1992) Inhibition of neutrophil superoxide production by human plasma alpha 1-antitrypsin. *FEBS Lett.* **300,** 21–24.
4. Heuertz, R. M., Piquette, C. A., and Webster, R. O. (1993) Rabbits with elevated serum C-reactive protein exhibit diminished neutrophil infiltration and vascular permeability in C5a-induced alveolitis. *Am. J. Pathol.* **142,** 319–328.
5. Libert, C., Brouckaert, P., and Fiers, W. (1994) Protection by alpha 1-acid glycoprotein against tumor necrosis factor-induced lethality. *J. Exp. Med.* **180,** 1571–1575.
6. Libert, C., Van Molle, W., Brouckaert, P., and Fiers, W. (1996) alpha1-Antitrypsin inhibits the lethal response to TNF in mice. *J. Immunol.* **157,** 5126–5129.
7. Xia, D., and Samols, D. (1997) Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. *Proc. Natl. Acad. Sci. USA* **94,** 2575–2580.
8. Itoh, N., Kasutani, K., Muto, N., Otaki, N., Kimura, M., and Tanaka, K. (1996) Blocking effect of anti-mouse interleukin-6 monoclonal antibody and glucocorticoid receptor antagonist, RU38486, on metallothionein-inducing activity of serum from lipopolysaccharide-treated mice. *Toxicology* **112,** 29–36.
9. Michalska, A. E., and Choo, K. H. (1993) Targeting and germline transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc. Natl. Acad. Sci. USA* **90,** 8088–8092.
10. Masters, B. A., Kelly, E. J., Quaife, C. J., Brinster, R. L., and Palmiter, R. D. (1994) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc. Natl. Acad. Sci. USA* **91,** 584–588.
11. Dalton, T., Fu, K., Palmiter, R. D., and Andrews, G. K. (1996) Transgenic mice that overexpress metallothionein-I resist dietary zinc deficiency. *J. Nutr.* **126,** 825–833.
12. Kelly, E. J., Quaife, C. J., Froelick, G. J., and Palmiter, R. D. (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J. Nutr.* **126,** 1782–1790.
13. Satoh, M., Nishimura, N., Kanayama, Y., Naganuma, A., Suzuki, T., and Tohyama, C. (1997) Enhanced renal toxicity by inorganic mercury in metallothionein-null mice. *J. Pharmacol. Exp. Ther.* **283,** 1529–1533.
14. Satoh, M., Aoki, Y., and Tohyama, C. (1997) Protective role of metallothionein in renal toxicity of cisplatinum. *Cancer Chemother. Pharmacol.* **40,** 358–362.
15. Rofe, A. M., Barry, E. F., Shelton, T. L., Philcox, J. C., and Coyle, P. (1998) Paracetamol hepatotoxicity in metallothionein-null mice. *Toxicology* **125,** 131–140.
16. Kimura, T., Oguro, I., Kohroki, J., Takehara, M., Itoh, N., Nakanishi, T., and Tanaka, K. (2000) Metallothionein-null mice express altered genes during development. *Biochem. Biophys. Res. Commun.* **270,** 458–461.
17. Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdell, J. N., Yamamoto, R., and Mark, D. F. (1985) Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* **228,** 149–154.
18. Sherman, P. A., Laubach, V. E., Reep, B. R., and Wood, E. R. (1993) Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. *Biochemistry* **32,** 11600–11605.
19. Sugatani, J., Miwa, M., Komiyama, Y., and Murakami, T. (1993) Quantitative analysis of platelet-activating factor in human plasma. Application to patients with liver cirrhosis and disseminated intravascular coagulation. *J. Immunol. Methods* **166,** 251–261.
20. Pinckard, R. N., Farr, R. S., and Hanahan, D. J. (1979) Physicochemical and functional identity of rabbit platelet-activating factor (PAF) released in vivo during IgE anaphylaxis with PAF released in vitro from IgE sensitized basophils. *J. Immunol.* **123,** 1847–1857.
21. Lee, S. C., Chang, C. J., Lee, Y. M., Lei, H. Y., Lai, M. Y., and Chen, D. S. (1989) Molecular cloning of cDNAs corresponding to two genes of alpha 1-acid glycoprotein and characterization of two alleles of AGP-1 in the mouse. *DNA* **8,** 245–251.
22. Leist, M., Gantner, F., Bohlinger, I., Tiegs, G., Germann, P. G., and Wendel, A. (1995) Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am. J. Pathol.* **146,** 1220–1234.
23. Morikawa, A., Kato, Y., Sugiyama, T., Koide, N., Chakravortty, D., Yoshida, T., and Yokochi, T. (1999) Role of nitric oxide in lipopolysaccharide-induced hepatic injury in D-galactosamine-sensitized mice as an experimental endotoxic shock model. *Infect. Immun.* **67,** 1018–1024.
24. Libert, C., Van Molle, W., Brouckaert, P., and Fiers, W. (1995) Platelet-activating factor is a mediator in tumor necrosis factor/galactosamine-induced lethality. *J. Inflamm.* **46,** 139–143.
25. Van Molle, W., Denecker, G., Rodriguez, I., Brouckaert, P., Van-

denabeele, P., and Libert, C. (1999) Activation of caspases in lethal experimental hepatitis and prevention by acute phase proteins. *J. Immunol.* **163,** 5235–5241.

26. Costello, M., Fiedel, B. A., and Gewurz, H. (1979) Inhibition of platelet aggregation by native and desialised alpha-1 acid glycoprotein. *Nature* **281,** 677–678.
27. Piguet, P. F., Vesin, C., Ryser, J. E., Senaldi, G., Grau, G. E., and Tacchini-Cottier, F. (1993) An effector role for platelets in systemic and local lipopolysaccharide-induced toxicity in mice, mediated by a CD11a- and CD54-dependent interaction with endothelium. *Infect. Immun.* **61,** 4182–4187.
28. Bellezzo, J. M., Leingang, K. A., Bulla, G. A., Britton, R. S., Bacon, B. R., and Fox, E. S. (1998) Modulation of lipopolysaccharide-mediated activation in rat Kupffer cells by antioxidants. *J. Lab. Clin. Med.* **131,** 36–44.
29. Kroncke, K. D., Fehsel, K., and Kolb-Bachofen, V. (1997) Nitric oxide: Cytotoxicity versus cytoprotection—how, why, when, and where? *Nitric Oxide* **1,** 107–120.
30. Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269,** 4705–4708.
31. Abdel-Mageed, A. B., and Agrawal, K. C. (1998) Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. *Cancer Res.* **58,** 2335–2338.
32. Sakurai, A., Hara, S., Okano, N., Kondo, Y., Inoue, J., and Imura, N. (1999) Regulatory role of metallothionein in NF-kappaB activation. *FEBS Lett.* **455,** 55–58.
33. Jaeschke, H., Farhood, A., and Smith, C. W. (1991) Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am. J. Physiol.* **261,** G1051–G1056.
34. Thornalley, P. J., and Vasak, M. (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta.* **827,** 36–44.
35. Quesada, A. R., Byrnes, R. W., Krezoski, S. O., and Petering, D. H. (1996) Direct reaction of H_2O_2 with sulfhydryl groups in HL-60 cells: zinc-metallothionein and other sites. *Arch. Biochem. Biophys.* **334,** 241–250.
36. Jaeschke, H., Ho, Y. S., Fisher, M. A., Lawson, J. A., and Farhood, A. (1999) Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. *Hepatology* **29,** 443–450.