Endotoxemia in Transgenic Mice Overexpressing Human Glutathione Peroxidases

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Abstract—In response to endotoxemia induced by administration of lipopolysaccharide, a complex series of reactions occurs in mammalian tissues. During this inflammation response, cells produce different mediators, such as reactive oxygen species, a number of arachidonic acid metabolites, and cytokines. The reactive oxygen species thus generated have been suggested to produce tissue injury as a result of macromolecular damage or by interfering with regulatory processes. They may also act as important signaling molecules to induce redox-sensitive genes. We report here that transgenic mice overexpressing 2 major forms of human glutathione peroxidases (GPs), intra- and extracellular GP, are able to modulate host response during endotoxemic conditions. We show that these animals have a decreased hypotension and increased survival rate after administration of a high dosage of lipopolysaccharide. Overexpression of GPs alters vascular permeability and production of cytokines (interleukin-1β and tumor necrosis factor-α) and NO, affects arachidonic acid metabolism, and inhibits leukocyte migration. These results suggest an important role for peroxides in pathogenesis during endotoxemia, and GPs, by regulating their level, may prove to be good candidates for antioxidant therapy to protect against such injury. (Circ Res. 2000;87:289-295.)

Key Words: endotoxemia ■ oxidative stress ■ glutathione peroxidase

Lipopolysaccharides (LPSs) are important mediators of multiple organ failure resulting from severe infection or sepsis caused by Gram-negative bacteria.1 Their release during infection induces a number of pathophysiologic reactions leading to cardiovascular failure and damage to numerous organs.2 There is experimental evidence that suggests oxidative damage in pathogenesis of endotoxemia.3 To evaluate the role of glutathione peroxidase (GP) in protection against endotoxemia in vivo, we have used transgenic mice overexpressing human extracellular (GPxP) and intracellular human GPx1 (strain 23) and their normal littermates were bred in our CBA/J background overexpressing human GPxP (strain 17) and human GPx1 (strain 23) and their normal littermates were bred in our facility. We described the generation of these mice previously.4 For survival studies, the mice were injected with LPS at a dose of 25 mg/kg (Escherichia coli serotype 0111:B4; Sigma). Arterial blood pressure and heart rate were measured using the Kent tail blood pressure system. For characterization of cytokine production and leukocyte distribution, blood was collected from the retro-orbital cavity of a group of animals dosed with LPS (16 mg/kg of body weight). Plasma was analyzed for cytokine (tumor necrosis factor-α [TNF-α] and interleukin-1β [IL-1β]) and nitric oxide production with ELISA kits from R&D Systems. Vascular permeability was determined by assessing tissue accrual of Evan’s Blue, as described previously.5 16 hours after PBS or LPS injection, the animals were administered 25 mg/kg Evan’s Blue by tail vein injection, and after 10 minutes the organs were harvested. Dye content was calculated by standard curves and referred to the dry weight of the tissue. For bronchoalveolar lavage (BAL), the animals were anesthetized and PBS (pH 7.0) was injected via catheter.

Hematology

Blood cells were analyzed on an EPICS Profile flow cytometer (Coulter Electronics, Inc). Leukocyte differentials were gated using forward light scatter and side scatter.

Myeloperoxidase (MPO) Assay

The lung extracts were assayed for MPO activity by a spectrophotometric assay according to the method of Goldblum et al.6

Measurement of Lipid Peroxides (LPOs) and GP Activity

LPOs were measured using LPO-586 colorimetric assay kit (R&D Systems). GP activity was measured by a coupled enzyme assay.4

Materials and Methods

Animals and Treatments

Heterozygous transgenic mice 5 to 6 months old in a C57BL/6×CBA/J background overexpressing human GPxP (strain 17) and human GPx1 (strain 23) and their normal littermates were bred in our facility. We described the generation of these mice previously.4

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Lactate Dehydrogenase and Nitrite/Nitrate Assay
Plasma and BAL nitrite/nitrate levels were measured in normal and transgenic mice after LPS administration using the R&D Systems NO$_7$/NO$_5$ assay kit.

Inducible NO Synthase (iNOS) Assay and Western Blot Analysis
iNOS activity was measured in cellular homogenates using an NO synthase assay kit based on conversion of [$^3$H]arginine to citrulline (Calbiochem) according to the manufacturer’s protocol. For protein detection, aliquots of the whole cellular extracts containing 5 μg of protein each were denatured and separated on 7.5% polyacrylamide minigels. After transfer to nitrocellulose membrane, iNOS protein was detected using rabbit anti-mouse iNOS antibodies (sc-650, Santa Cruz Biotechnology) and the Phototope-HRP Western blot detection kit (BioLabs).

Isolation of Intraperitoneal Macrophages, Blood Neutrophils, and Platelets
Intraperitoneal macrophages were isolated as described earlier.$^7$ Platelets were purified from mouse blood obtained by cardiac puncture, as described by Hill et al.$^4$ Mouse blood neutrophils were purified by using neutrophil isolation medium (NIM-2) density gradient medium (Cardinal Associates Inc).

Measurement of Neutrophil Adhesion In Vitro
Neutrophil adhesion was measured according to Vasselon et al$^9$ within 24-well plates precoated with laminin and fibronectin (Becton Dickinson). The percentage of cells remaining adherent after 30 minutes of incubation was evaluated using the CyQUANT assay kit (Molecular Probes, Inc).

Leukotriene B$_4$ (LTB$_4$) and 12-HETE Production in Blood Plasma During Endotoxemia
Eicosanoids were extracted from blood plasma by Sep-Pak C-18 cartridge$^{10}$ and measured using ELISA kits (Amersham and PerSep-tive Biosystems).

Analysis of Eicosanoids Produced by Platelets Using Precolumn
Extraction/Reversed-Phase HPLC
Platelets (5×10$^9$) from normal and transgenic mice were incubated for 20 minutes in 1 mL of Tyrode-HEPES buffer in the presence of 50 mmol/L and 10 mmol/L of unlabeled and $^{14}$C-labeled arachidonic acid (57 mCi/mmol), respectively, at 37°C. The reaction was stopped by adding 1 mL of cold methanol. Eicosanoids were quantified by precolumn extraction/reversed-phase HPLC.$^{11}$

Results
GP Activity in Leukocytes and Platelets
We have previously reported the levels of GP activity in tissues and blood plasma of both the transgenic mouse strains used in our experiments.$^3,12$ In GPx1 mice, GP activities were higher in brain, muscle, liver, kidney, and lung than in the corresponding tissues of nontransgenic animals. In GPxP mice, increase of activity was observed only in blood plasma and kidney, which correlates with normal distribution of this enzyme activity in mice. Because leukocytes as well as platelets are active participants in the inflammatory response during endotoxemia, we also measured activity of GP in these cells. No statistically significant difference was observed in GP activity in neutrophils among Gpx1, GpxP, and nontransgenic mice, whereas peritoneal macrophage level of GP was 35% higher in GpxP and 38% higher in Gpx1 animals as compared with that in normal mice (1123.5±108.7, 1143.5±67.0, and 830.0±62.1 mU/mg, respectively). Similar data were obtained by using purified blood monocytes. Interestingly, in platelets, increase of activity was even higher, 62% in Gpx1 and 49% in GpxP in comparison with normal animals (62.5±2.6, 59.8±7.1, and 39.92±4.1 mU/mg, respectively).

LPS-Induced Lethality and Blood Pressure in Nontransgenic and Transgenic Mice With Human GPs
Lethal endotoxic shock was induced in mice by administering LPS at a dosage of 25 mg/kg. More than 40% of GpxP mice and 70% of Gpx1 mice survived, whereas only 20% of nontransgenic animals survived in the experiment with GpxP mice and 30% survived in the experiment with Gpx1 mice (Figures 1A and 1B). Because the strains of transgenic mice with different transgenes show similar phenotypes, these data indicate that overexpression of GPs leads to the attenuation of death and better survival of mice at a high dosage of LPS. Histopathological analysis of liver, lung, heart, and kidney sections at 12 and 18 hours after LPS injection as well as necropsies of dead animals indicate only mild histopathological alterations in all groups, which cannot be associated with morbidity and mortality in these animals.

Several lines of evidence suggest that hypotension, cardiorepression, and vascular hyporeactivity that accompany endotoxemia in patients and experimental animals might result in organ hypoperfusion and potential circulatory collapse leading to high mortality.$^{13}$ We measured mean arterial blood pressure in the conscious transgenic and normal mice before and after administration of LPS. Results indicate that LPS injection caused a progressive fall in blood pressure (Figure 1C). In a group of normal mice after 15 to 16 hours, this led to a very low level of blood pressure (40±3.8 mm Hg) followed by death of most of the animals. In contrast, blood pressure of Gpx1 and GpxP mice showed signs of stabilization at this time point, with level of blood pressure ~60 mm Hg. The next decline of blood pressure in the GP transgenic mice was observed after 40 hours (data not shown), which is in good correlation with survival curves. No differences in alteration of heart rate during endotoxemia were observed between the groups.

Effect of Endotoxemia on GP Activity
To evaluate the effect of LPS administration on GP activity in different organs and cells of normal and transgenic mice, heart, kidney, lung, liver, platelets, and macrophages were isolated from animals at 8 and 18 hours after LPS administration (25 mg/kg). Measurements indicate that there was no change in the GP activity in heart, kidney, liver, and platelets from normal and transgenic mice at these time points. At 18 hours, a slight increase of GP activity (30%) was observed in the lungs of normal mice (92±11 mU/mg before and 130±12 mU/mg after LPS administration; P<0.05). A moderate decrease of GP activity in lung was observed in GpxP and Gpx1 mice (118±21 and 128±15 mU/mg before and 105±9 and
In macrophages, GP activity dropped at 18 hours in all animal groups to 50% to 60%, although it was still 43% and 34% higher in GPx1 and GPxP mice than in normal animals (682 ± 109 [P < 0.05], 582 ± 29 [P < 0.05], and 388 ± 52 mU/mg).

These data indicate that overall GP activity in most of the organs was not affected by this dosage of LPS and are in agreement with data on GP measurements in several organs by Yoshikawa et al. Changes in GP activity in lungs of normal animals probably reflect compensatory increase of the enzymatic activity in this group of animals, which undergo higher stress during endotoxemia as compared with transgenic mice.

Effect of GP Overexpression on NO Production During Endotoxemia and Vascular Permeability

Massive release of NO during endotoxemia was shown to contribute to different cardiovascular abnormalities, including hypotension and lowered peripheral resistance. Administration of LPS to normal and transgenic mice led to a sharp increase of nitrite/nitrate in the plasma (Figure 2A). Importantly, the levels of nitrite/nitrate at 8 and 16 hours were 30% to 40% less in both GPx1 and GPxP mice. We also measured the level of nitrite/nitrate in BAL, in which formation of NO is thought to be involved in development of acute lung injury during endotoxemia and sepsis. The level of induction of nitrite/nitrate in BAL at 8 hours was 2-fold less in transgenic mice; however, at 16 hours it remained at a lower level only in the GPxP mice (Figure 2B). Because iNOS induction is primarily responsible for the production of large amounts of NO during endotoxemia, iNOS activity was measured in normal and transgenic mice before and after LPS administration. Data shown in Figure 2C indicate significantly higher iNOS activity in macrophages from normal mice in comparison with GPx1 and GPxP mice. These measurements correlate with increased amounts of iNOS protein detected by Western blotting in the same cells (Figure 3).

Hypotension has also been attributed to a direct action of LPS on endothelial cells leading to an increase in vascular permeability. Vascular permeability, evaluated by the Evan’s...
Blue dye extravasation method, shows a significant increase in all tested organs of normal mice (35.15 ± 12.3 and 319.8 ± 48 μg in kidney, 29.12 ± 11.3 and 251.5 ± 35.5 μg in lung, 15.6 ± 2.8 and 206.7 ± 43 μg in liver, and 8.7 ± 2.1 and 12 ± 5.6 μg in heart of Evan’s Blue per gram dry tissue after PBS or LPS administration, respectively; n = 4), whereas in GP transgenic mice, much lower dye extravasation in kidney, liver, and lung, but not heart, was observed. For example, measurements of Evan’s Blue dye in those tissues from GPx1 mice after LPS administration were as follows: 96.8 ± 29.1 μg in kidney, 58.2 ± 18.3 μg in liver, and 54.3 ± 15.3 μg in lung (P < 0.05 versus nontransgenic mice) and 15.2 ± 6 μg in heart of Evan’s Blue per gram dry tissue (n = 4; Evan’s Blue extravasation in GPs and normal mice was similar after injection of PBS). Similar data were obtained for GPxP mice. These results indicate a dramatic improvement of vascular integrity during endotoxemia by GP overexpression.

Overexpression of GPs Leads to the Modulation of the Level of Lipid Peroxidation During Endotoxemia

Levels of LPOs during LPS-induced endotoxemia were measured in the liver, kidney, and blood of nontransgenic, GPx1, and GPxP mice. The presence of both of the human enzymes decreases the level of lipid peroxidation in tissues of tested organs only at 4 hours after LPS administration (Figures 4A and 4B). No difference was observed in LPOs between nontransgenic and GP mice in blood plasma (Figure 4C). These data indicate that the antioxidant effect of GP overexpression occurs very early during the inflammatory response to LPS administration.

Induction of the TNF-α and IL-1β in GP Transgenic Mice

Cytokine production is an important early response of an organism to endotoxemia. We have measured levels of TNF-α and IL-1β in nontransgenic, GPx1, and GPxP mice under endotoxemic conditions. Administration of LPS causes a sharp increase of TNF-α production in all groups of mice, with a peak at ~90 minutes after injection (Figure 5A). Surprisingly, the level of TNF-α induction was 3 times higher in GPx1 mice and 80% higher in GPxP mice than in nontransgenic animals. After 6 hours, the level of this cytokine returns to normal in all of the mice. The quantity of IL-1β in blood, in contrast to TNF-α, at 3 hours was 25% and 80% higher in nontransgenic mice than in GPx1 and GPxP, respectively (Figure 5B). Similar differences were observed at 6 hours after LPS administration as well. These data indicate that GP overexpression differentially influences cytokine production.
Leukocyte Migration Under Endotoxic Conditions Is Inhibited in GP Transgenic Mice

Because leukocyte recruitment is an important feature of severe endotoxemia, we determined blood leukocyte differentials of transgenic animals after LPS administration. After a sharp decline of circulating neutrophil counts in all groups of animals, significantly higher levels of these leukocytes were observed in transgenic mice after 6 hours (Figure 6A). These data correlate with MPO activities (Figure 6D). Monocytopenia was detected in GPx1 and nontransgenic mice at 2 hours, which then gradually changed to an elevated level in the former (Figure 6B). In GPxP mice, a 4-time increase in comparison with nontransgenic mice was seen at 2 hours, whereas at 24 hours it decreased to a similar level. Severe absolute lymphopenia was observed in all groups of mice (Figure 6C), although at 24 hours, the level of lymphocytes was higher in GP mice. These data suggest that the level of GP activity, both intracellular and extracellular, modulates recruitment of leukocytes.

The basic adhesion properties of neutrophils from transgenic and nontransgenic mice were similar (tested by binding to laminin- and fibronectin-coated plates). By Northern blot analysis, we also demonstrated that the expression of at least 1 receptor molecule, intercellular adhesion molecule 1, which mediates extravagation of leukocytes in an inflammatory tissue, was equal in all of the tested animal groups (not shown). Therefore, differences observed in leukocyte behavior were probably due to the level of their activation in vivo.

GP Overexpression Decreased Production of Several Important Eicosanoid Metabolites in the Blood of Animals During Endotoxemia as Well as in Purified Activated Platelets

Along with ROS, eicosanoids belong to the group of proinflammatory agents that cause systemic hypotension and attract, activate, and promote adherence of neutrophils. For example, LTB4 and 12-HETE stimulate neutrophil chemokinesis, chemotactic aggregation, adherence, and potential inflammatory reactions, attracting neutrophils to the place of their involvement.17,18 Because GP is known to influence cyclooxygenase and lipoxygenase activity,19,20 we have measured LTB4 and 12-HETE in plasma of animals after LPS administration. At the 6-hour time point, an almost 30% decrease in LTB4 production was observed in GP mice in comparison with nontransgenic mice (Figure 7A). The level of 12-HETE was ~1.6 to 2.1 times lower in plasma of GPx1 and GPxP mice in comparison with the nontransgenic animals at the 6-hour time point (Figure 7B).

Along with leukocyte 12-LPO, platelet-type 12-LPO is a major source of 12-HETE. To test the hypothesis that platelets, because of the different level of GP, were able to decrease 12-HETE production in transgenic mice, we purified these cells and measured the release of arachidonic acid metabolites. Two major products were detected (12-HETE and 12(S)-hydroxyheptadecatrienoic acid [12-HHTrE]). The level of production of these eicosanoids was inhibited in platelets of GP mice by 50% and 40% for 12-HETE and 12-HHTrE, respectively (Figures 8A and 8B). These data correlate with similar levels of GP overexpression in platelets from both types of transgenic mice. 12-HHTrE is one of the major products of the cyclooxygenase-thromboxane synthase pathway in activated platelets. It is formed along with thromboxane A2, a very potent aggregatory agent.

Discussion

It has become apparent that ROS may be important mediators of cellular injury during endotoxemia either as a result of macromolecular damage or by interfering with extracellular and intracellular regulatory processes. Studies presented here show that transgenic mice overexpressing GPs exhibit decreased sensitivity to endotoxic shock induced by a large dosage of LPS. Our data indicate that this effect correlates with significantly higher blood pressure and vascular perme-
ability in these animals in comparison with nontransgenic mice, which suggests reduced circulatory failure by GP overexpression. The role of GPx1 in another model of inflammatory injury was also emphasized by Jaeschke et al., who observed increased sensitivity of GPx1 knockout animals to hepatocellular toxicity, induced by galactosamine/endotoxin administration.

Measurements of the inflammatory mediators, which are thought to play a critical role in the pathological response during endotoxic shock in normal and transgenic mice, provides several potential mechanisms to explain the influence of GP overexpression on animal survival. We observed an inhibitory effect of GP on the increase of vascular permeability and level of NO in blood and BAL of animals after LPS injection, which are essential for the development of circulatory failure, and therefore a significant effect on survival during endotoxemia was observed.

In conclusion, the overall picture that emerges during endotoxemia in GP transgenic mice is very complex. Only 1 single event or mediator is unlikely to be responsible for the altered response in these animals, but a multitude of factors may contribute to the observed phenotype. We hypothesize that GP overexpression is able to influence extracellular and intracellular redox balance, leading to the modulation of NO, arachidonic acid metabolites, and cytokines. Those alternations lead to the inhibition of hypotension and improved circulatory failure, and therefore a significant effect on survival during endotoxemia was observed.

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