Am J Physiol Lung Cell Mol Physiol
279: L390–L398, 2000.

Dietary glycine blunts lung inflammatory cell influx following acute endotoxin

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Received 2 November 1999; accepted in final form 28 March 2000

Wheeler, Michael D., Michelle L. Rose, Shunhei Yamashima, Nobuyuki Enomoto, Vitor Seabra, Jonathan Madren, and Ronald G. Thurman. Dietary glycine blunts lung inflammatory cell influx following acute endotoxin. *Am J Physiol Lung Cell Mol Physiol* 279: L390–L398, 2000.—Mortality associated with endotoxin shock is likely mediated by Kupffer cells, alveolar macrophages, and circulating neutrophils. Acute dietary glycine prevents mortality and blunts increases in serum tumor necrosis factor-α (TNF-α) following endotoxin in rats. Furthermore, acute glycine blunts activation of Kupffer cells, alveolar macrophages, and neutrophils by activating a glycine-gated chloride channel. However, in neuronal tissue, glycine rapidly downregulates chloride channel function. Therefore, the long-term effects of a glycine-containing diet on survival following endotoxin shock were investigated. Dietary glycine for 4 wk improved survival after endotoxin but did not improve liver pathology, decrease serum alanine transaminase, or effect TNF-α levels compared with animals fed control diet. Interestingly, dietary glycine largely prevented inflammation and injury in the lung following endotoxin. Surprisingly, Kupffer cells from animals fed glycine for 4 wk were no longer inactivated by glycine in vitro; however, isolated alveolar macrophages and neutrophils from the same animals were sensitive to glycine. These data are consistent with the hypothesis that glycine downregulates chloride channels on Kupffer cells but not on alveolar macrophages or neutrophils. Importantly, glycine diet for 4 wk protected against lung inflammation due to endotoxin. Chronic glycine improves survival by unknown mechanisms, but reduction of lung inflammation is likely involved.

endotoxin shock; Kupffer cells; alveolar macrophages; neutrophils; tumor necrosis factor-α

INFLAMMATORY CELLS SUCH AS alveolar macrophages and Kupffer cells, the resident hepatic macrophages, are thought to play critical roles in organ failure due to endotoxin (3, 31). Both cell types produce tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and other toxic mediators that lead to tissue injury (11). Serum TNF-α is an important mediator because it is involved in signaling between several cell types and may potentiate macrophage activation and tissue injury following endotoxin (4, 21). The precise role of TNF-α due to endotoxin shock and the primary source of serum TNF-α are still largely unknown; however, injection of recombinant TNF-α mimics most of the effects of endotoxin (22).

Importantly, it was reported recently that administration of dietary glycine for 3 days blunted the increase in serum TNF-α after endotoxin injection, which markedly improved survival and minimized liver and lung injury due to endotoxin in rats (6). Furthermore, glycine directly inactivated Kupffer cells and alveolar macrophages via activation of a glycine-gated chloride channel (7, 29). Therefore, it was concluded that glycine protects the liver and the lung from injury due to endotoxin by inactivating Kupffer cells and alveolar macrophages by blunting the production of TNF-α (7). The pharmacological properties of the glycine-gated chloride channel in the Kupffer cell parallel those described for the spinal cord glycine-gated chloride channel (7, 15). Glycine activates the channel, and chloride enters the cells and hyperpolarizes the plasma membrane. This prevents lipopolysaccharide (LPS)-induced increases in intracellular calcium via voltage-dependent calcium channels. Furthermore, recent molecular evidence showed that the glycine-gated chloride channel exists in Kupffer cells with molecular properties similar to the channel in spinal cord (27). Other white blood cells such as neutrophils and lymphocytes recently have also been shown to possess glycine-gated chloride channels (20, 28, 30).

It was hypothesized that the receptor in the Kupffer cell and alveolar macrophage would also be downregulated due to chronic exposure to glycine, like the glycine-gated chloride channel in the central nervous system (15). Therefore, the purpose of this study was to evaluate the effects of long-term feeding of glycine in an endotoxin shock model on Kupffer cell, alveolar macrophage, and neutrophil activation. Herein, it is reported that 4 wk of dietary glycine improved survival following endotoxin shock and indeed caused downregulation of the glycine receptor on Kupffer cells. However, long-term dietary glycine treatment had no

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effect on the glycine receptor in alveolar macrophages or neutrophils. Importantly, glycine blocked the accumulation of neutrophils in the lung.

MATERIALS AND METHODS

Animals and diet. Male Sprague-Dawley rats weighing 200–250 g were given free access to a AIN-76 synthetic powdered diet containing 5% glycine or casein as a nitrogen-balanced control diet for up to 32 wk (17). Diets were a generous gift from Novartis Nutrition. Animals were individually caged under identical housing conditions, given humane care, and maintained in compliance with institutional guidelines.

Endotoxin treatment. After dietary treatment, various amounts of LPS (1–40 mg/kg; *Escherichia coli* serotype 0111:B4; Sigma, St. Louis, MO) in saline were injected intravenously via the tail vein, and survival was assessed over 24 h. At the end of experiments, serum and tissue were collected under pentobarbital sodium (75 mg/kg; Nembutal; Abbott Laboratories, North Chicago, IL) anesthesia. All treatments with LPS were performed simultaneously in both glycine- and casein-fed animals. Liver and lung specimens were fixed in phosphate-buffered Formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin for histological evaluation.

Kupffer cell preparation and culture. Kupffer cells were isolated by collagenase digestion and differential centrifugation with Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere with slight modifications (19). Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) at 37°C for 5 min at a flow rate of 40 ml/min. Subsequently, perfusion was with HBSS containing 0.025% collagenase IV (Sigma) at 37°C for 5 min at a flow rate of 26 ml/min. After the liver was digested, it was excised and minced in collagenase buffer. The suspension was filtered through nylon gauze, and the filtrate was centrifuged at 450 *g* for 10 min at 4°C. Cells were resuspended in buffer, parenchymal cells were removed by centrifugation at 50 *g* for 3 min, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of 50:25% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) in HBSS at 1,000 *g* for 15 min. The Kupffer cell fraction was collected and washed with buffer. Cells were seeded onto 25-mm glass coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate) at 37°C in a 5% CO_2 atmosphere. After 1 h of incubation, the medium was exchanged with fresh medium to remove nonadherent cells (i.e., stellate and endothelial cells). Cells were cultured for 24–48 h before experiments. Purity was verified by uptake of FITC-labeled latex beads.

Alveolar macrophage preparation and culture. Alveolar macrophages were isolated by bronchoalveolar lavage as previously described (2). Briefly, the trachea was cannulated, and the lungs were lavaged four times with 10 ml of phosphate-buffered saline warmed to 37°C. Cells were centrifuged at 500 *g*, and the pellet was resuspended in DMEM. Yield of alveolar macrophages following lavage was greater than 8×10^6 cells and was determine to be greater than 95% viable by trypan blue exclusion. Alveolar macrophages were seeded onto 25-mm glass coverslips and cultured as described above for Kupffer cells.

Neutrophil preparation and culture. Saline (35 ml) containing 1% oyster glycogen type II (Sigma) was administered intraperitoneally to male Sprague-Dawley rats (300–350 g) under light anesthesia with methoxyflurane. Four hours later, animals were reanesthetized and exsanguinated, and cells in the peritoneum were removed by lavage with saline (35 ml) containing heparin (1,000 U/l). The suspension was centrifuged at 500 *g* for 7 min, and the pellet was resuspended in 0.15 M NH_4Cl to lyse erythrocytes. Cells were pelleted again by centrifugation at 500 *g* for 7 min. The cell pellet was suspended in RPMI 1640 containing L-glutamine (1 mM), 10% heat-inactivated fetal bovine serum, penicillin (10 IU/ml), and streptomycin (10 μg/ml). Cells (0.5×10^6 cells) were seeded onto 25-mm glass coverslips and incubated for 1 h at 37°C. The medium was then replaced with fresh medium, and cells were cultured for 24 h at 37°C. Neutrophils were identified microscopically by the lobular shape of their polymorphic nuclei and were >90% in all cell preparations.

Measurement of intracellular calcium concentration. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) of single cells were measured fluorometrically using the calcium indicator fura 2 (7). Briefly, cells were plated on glass coverslips at a density of 3.0×10^5 cells/coverslip and were incubated in 2 ml of HBSS [(in mM) 15 HEPES, 110 NaCl, 5 KCl, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 0.8 $MgSO_4 \cdot 7H_2O$, 1.25 $CaCl_2 \cdot 2H_2O$, 4 $NaHCO_3$, and 5.6 glucose] containing 5 μM fura 2 acetoxymethyl ester (Molecular Probes, Eugene, OR) at room temperature for 30 min. After being loaded, cells were rinsed and placed in a measurement chamber with HBSS buffer at room temperature. A microspectrofluorometer (Photon Technology, South Brunswick, NJ) attached to an inverted microscope (Diaphot, Nikon, Japan) was used to monitor changes in intracellular calcium. Changes in fluorescent intensity of fura 2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were recorded continuously in individual cells. The ratio of emission at 340 and 380 nm was determined, and the corresponding value of $[Ca^{2+}]_i$ was calculated using the relationship

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)(F_0/F_s)$$

where F_0/F_s is the ratio of fluorescent intensities in buffers containing 3 mM EGTA and 1 μM ionomycin ($[Ca^{2+}]_{min}$) or 10 mM Ca^{2+} and 1 μM ionomycin ($[Ca^{2+}]_{max}$). R is the measured ratio of fluorescent intensities at excitation wavelengths of 340 and 380 nm, and R_{max} and R_{min} are values of R at $[Ca^{2+}]_{max}$ and $[Ca^{2+}]_{min}$, respectively. A dissociation constant (K_d) of 135 nM was used.

Clinical chemistry. Serum was collected 8 h after injection of saline or endotoxin (10 mg/kg). Serum alanine transaminase and alkaline phosphatase levels were measured by standard enzymatic methods (Sigma). The peak in serum transaminases following LPS occurred 6–12 h after injection (data not shown).

Measurement of serum TNF-α levels. Rats were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Mundelein, IL), and an intravenous catheter was inserted into the femoral vein for serial blood sampling. Whole blood (200 μl) was collected at each time point coincident with the injection of 200 μl of lactated Ringer solution. Aprotinin (75 μl; Sigma) was added to the serum immediately after collection, and samples were stored at −80°C until TNF-α measurement. Serum TNF-α was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, MA).

Glycine measurement. Rat serum and lavage fluid were collected, and the glycine concentration was determined (12, 13). For the collection of lung fluid, the lungs were lavaged with 10 ml/kg body wt of neutral-buffered saline. In brief, glycine was extracted and benzoylated, and the resulting

hippuric acid was extracted and dried in a nitrogen stream. The concentration of a colored conjugate of hippuric acid with dimethylaminobenzaldehyde was determined spectrophotometrically at 458 nm.

Tissue preparation and histological analysis. Liver and lung specimens were harvested when the rats were killed, fixed in 10% phosphate-buffered Formalin for 24 h, and mounted in paraffin. Sections (8 μm) were cut and counterstained with eosin and hematoxylin. The number of neutrophils per high-power field (×400) was counted based on nuclear shape in 10 random fields.

Statistical analysis. All results are expressed as means ± SE. Statistical differences between means were determined using analysis of variance followed by Tukey's post hoc analysis or by Student's *t*-test as appropriate. $P < 0.05$ was selected before analysis to reflect statistical significance.

RESULTS

Routine parameters. Sprague-Dawley rats were given free access to AIN-76 synthetic powdered diet containing 5% glycine or casein as a nitrogen-balanced control diet. The body weight and food consumption were measured daily (Table 1). Body weights of animals and food consumption in both groups over 4 wk were not significantly different. Importantly, glycine-fed rats had blood glycine concentrations nearly eight times higher than those in the control group (Table 1).

Effect of long-term glycine diet on survival after LPS injection. To evaluate the efficacy of long-term dietary glycine, animals received either powdered diet containing 5% glycine or nitrogen-balanced casein control diet for 4 wk. After dietary treatment, an intravenous injection of LPS (5–40 mg/kg) was given via the tail vein, and survival after 24 h was assessed. All animals survived after the injection of 5 mg/kg of LPS. However, mortality rates of 20, 80, 100, and 100% were observed with 10, 20, 30, and 40 mg/kg of LPS, respectively, in animals that received control diet for 4 wk (Fig. 1). In animals that received glycine diet, mortality after 10 and 20 mg/kg of LPS was completely prevented and slightly attenuated in animals given 30 mg/kg of LPS compared with casein-fed control animals. These data demonstrate that glycine is protective against of LPS-induced mortality even after chronic (>4 wk) consumption of glycine.

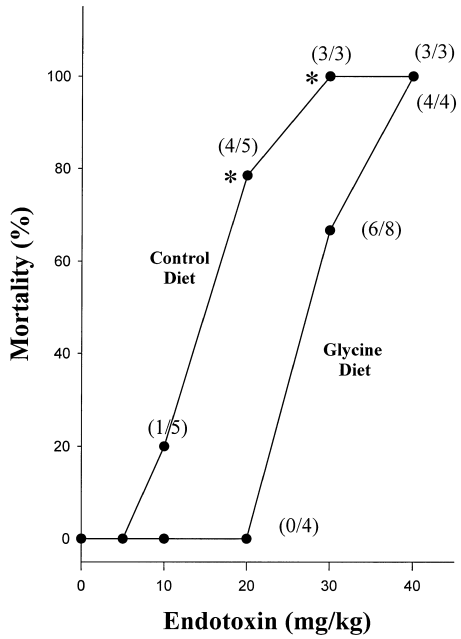

Fig. 1. Long-term dietary glycine improves survival after endotoxin shock. Wistar rats fed either casein diet or 5% glycine diet for 4 wk were injected with lipopolysaccharide (LPS, 0–40 mg/kg iv) in saline, and survival was assessed after 24 h. Data are expressed as percent mortality due to endotoxin. Fractions indicate the numbers of animals that died per total number of animals in each group. Statistical analysis was performed using Fisher's exact test (*$P < 0.05$).

Table 1. *Summary of body weight, food consumption, and blood glycine concentrations after 4 wk of dietary glycine*

	Control Diet	Glycine Diet
Body weight before feeding, g	222 ± 4	206 ± 7
Body weight after 4 wk of diet, g	354 ± 12	342 ± 24
Food consumption, $g \cdot kg^{-1} \cdot day^{-1}$	7.6 ± 0.5	7.8 ± 0.4
Blood glycine concentration, mM	0.2	1.2
Alveolar lavage glycine concentration, mM	<0.1	0.6

Values are means ± SE; $n = 4$ rats. Male Sprague-Dawley rats were fed an AIN-76-based diet containing 5% glycine or a nitrogen-balanced control diet containing casein for 4 wk. Animals were monitored daily for body weight gain and food consumption. After 4 wk of dietary treatment, blood and alveolar lavage fluid were collected and the glycine concentration was determined as described in MATERIALS AND METHODS.

Long-term glycine diet minimizes lung but not liver injury due to LPS. Because survival following LPS administration was improved after 4 wk of glycine, it was hypothesized that glycine protected the liver and lungs from injury due to endotoxin by inactivating Kupffer cells and alveolar macrophages, respectively. Accordingly, animals fed 5% glycine diet or casein diet for 4 wk were given 10 mg/kg of LPS, and liver and lung specimens were collected 24 h later. This dose of LPS was chosen because it was shown above to cause mortality only in 20% of control animals. LPS caused significant influx of lymphocytes and neutrophils in the livers of animals fed casein- and glycine-containing diets for 4 wk (Fig. 2). Mild liver necrosis was also observed in both the casein- and glycine-fed animals following injection of LPS. Serum alanine transaminase and alkaline phosphatase levels were measured in both casein- and glycine-fed animals 8 after LPS (Table 2). Values increased four- to sixfold in both groups. Furthermore, no significant differences in the increase in serum enzymes were observed between the groups.

In animals fed casein for 4 wk, lung specimens demonstrated hypercellularity of the alveolar wall due to inflammatory cell influx 24 h after LPS injection (Fig. 2*C*). Moreover, mild pathological alterations such as collapsed alveoli are also observed. However, glycine dramatically reduced these acute changes as well as blunted inflammatory cell influx

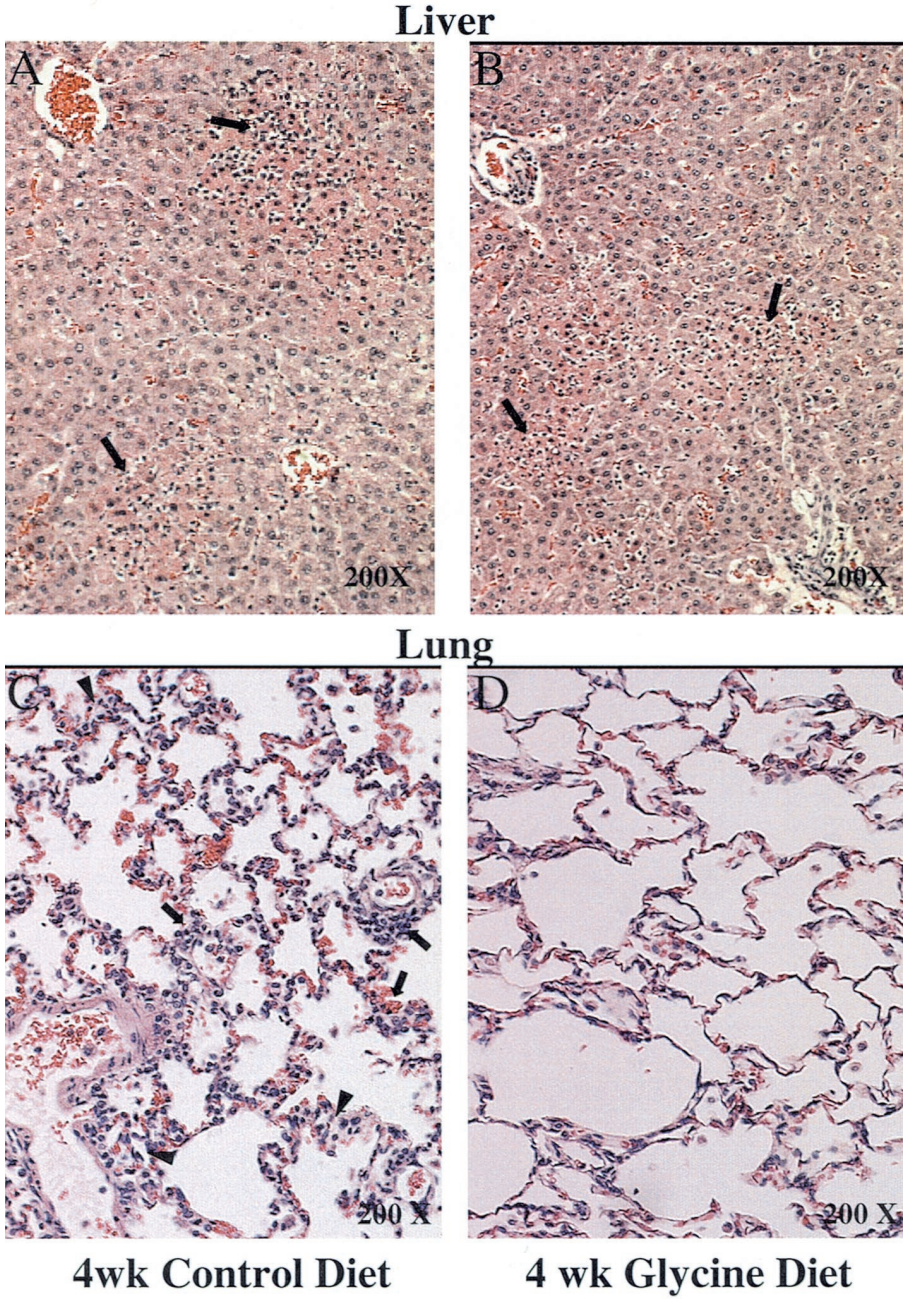

Fig. 2. Effect of long-term dietary glycine on liver and lung histology after endotoxin. Wistar rats fed either casein control diet (*A* and *C*) or 5% glycine-containing diet (*B* and *D*) for 4 wk were injected with 10 mg/kg of LPS. Livers and lungs were harvested 24 h after LPS injection. Arrowheads, collapsed alveoli or disrupted alveolar walls; arrows, infiltration. The photomicrographs are representative of 4 or more animals in each experiment. Original magnification, ×200.

in the lung (Fig. 2*D*). Wet lung weight was compared with dry lung weight 24 h after LPS as an index of lung injury (Table 2) (32). Lung wet-to-dry weight ratios increased significantly in control animals after LPS but not in glycine-fed animals. These data are consistent with the pathological changes observed in the lung following endotoxin. Moreover, in glycine-fed animals, the number of neutrophils 24 h after endotoxin (62 ± 4 cells/field) was significantly less than in casein-fed animals given endotoxin (142 ± 12 cells/field; $P < 0.05$, Student's *t*-test).

Effect of long-term dietary glycine on LPS-induced serum TNF-α levels. TNF-α has been hypothesized to mediate many symptoms observed in endotoxin shock (22, 23), and it was shown previously that 3 days of dietary glycine blunted the increase in serum TNF-α levels after LPS injection (6). To test the hypothesis that chronic dietary glycine would also blunt LPS-induced increases in TNF-α, animals fed 5% glycine or casein for 4 wk were injected with LPS (1 mg/kg), and TNF-α was measured in the serum (Fig. 3). TNF-α levels in animals fed casein rose to around 1,100 pg/ml within 60 min after injection of endotoxin and diminished slowly over the course of 4 h. TNF-α levels in animals fed glycine peaked near 950 pg/ml, values not significantly different from the casein group. These data are consistent with the hypothesis that the protective effect of long-term dietary glycine is not due to an effect of glycine on systemic TNF-α production.

Table 2. *Summary of liver injury and lung injury following endotoxin*

	Casein Diet		Glycine Diet	
	Saline	LPS	Saline	LPS
Serum alanine transaminase, U/l	23 ± 4	142 ± 15*	29 ± 9	145 ± 43*
Serum alkaline phosphatase, U/l	21 ± 5	69 ± 7*	20 ± 6	62 ± 8*
Liver weight/body weight, %	4.6 ± 0.2	5.3 ± 0.5	4.7 ± 0.3	5.4 ± 0.4
Lung wet-to-dry weight ratio	4.8 ± 0.2	6.0 ± 0.2*	4.7 ± 0.2	5.2 ± 0.2

Values are means ± SE of 3 individual experiments. Male Sprague-Dawley rats were fed diets containing casein or 5% glycine for 4 wk. Animals were injected with lipopolysaccharide (LPS; 10 mg/kg iv), serum was collected at 8 h, and liver and lung were collected 24 h after LPS. *$P < 0.05$ compared with saline controls, 2-way ANOVA followed by Tukey's post hoc analysis.

Effect of long-term dietary glycine on Kupffer cell activation. To test the hypothesis that long-term glycine feeding would prevent the activation of Kupffer cells due to LPS as in earlier short-term feeding studies (6), animals received either 5% glycine or casein control diet for 4 wk. Kupffer cells were isolated, and intracellular calcium was measured after addition of LPS (10 μg/ml) (Fig. 4). Kupffer cells from animals fed casein for 4 wk responded to LPS with a transient increase in intracellular calcium within 30 s after stimulation that reached nearly 350 nM and returned to basal levels within 2–3 min. When glycine (1 mM) was added acutely to Kupffer cells from casein-fed animals 3 min before LPS, the increase in $[Ca^{2+}]_i$ was blunted by nearly 70%, confirming earlier work with cells from naïve rats (7). In Kupffer cells from animals fed 5% glycine for 4 wk, LPS also caused a transient increase in calcium similar to the increase observed in cells from the casein controls. However, unlike Kupffer cells from the casein-fed controls, glycine added 3 min before LPS had no effect on the increase in intracellular calcium in cells from glycine-treated rats. Thus after long-term (>4 wk) dietary glycine treatment, glycine is unable to inactivate the Kupffer cell.

It was therefore hypothesized that long-term elevation of glycine in the blood desensitized the glycine receptor on the Kupffer cell. To test this hypothesis, Kupffer cells were isolated from animals fed either 5% glycine or casein diet for up to 8 wk, and intracellular calcium was measured as detailed above. After 2 wk of glycine diet, Kupffer cells were no longer inactivated by glycine, whereas Kupffer cells from casein-fed animals were (Fig. 5). These data support the hypothesis that elevated blood glycine causes a loss of function of the glycine-gated chloride channel in the Kupffer cell and that it occurs after about 2 wk.

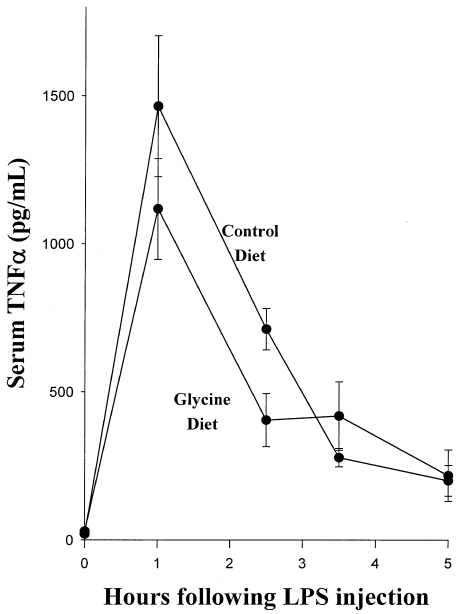

Fig. 3. Serum tumor necrosis factor-α (TNF-α) levels after LPS administration. Wistar rats were fed either casein or 5% glycine for 4 wk and injected with LPS (5 mg/kg iv). Serum was collected from a cannula in the femoral vein under pentobarbital sodium anesthesia (60 mg/kg) at 0, 60, 150, and 210 min after injection of LPS. TNF-α was measured using ELISA as described in MATERIALS AND METHODS, and data are expressed as means ± SE of 4 or more individual experiments. $P > 0.05$ by repeated-measures ANOVA.

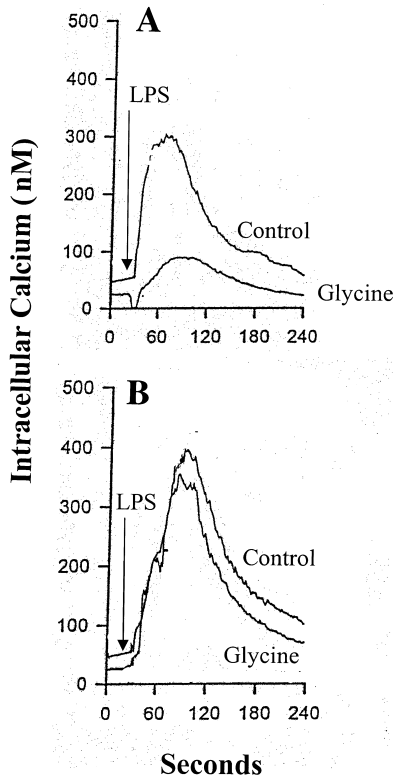

Fig. 4. Long-term dietary glycine downregulates glycine-gated chloride channel function in Kupffer cells. Intracellular calcium was measured in Kupffer cells isolated from Wistar rats fed either casein diet (*A*) or 5% glycine diet (*B*) for 4 wk as described in MATERIALS AND METHODS. Cells were incubated in Hanks' balanced salt solution (HBSS) or HBSS containing glycine (1 mM) for 3 min before addition of 10 μg/ml LPS. Data are representative traces from 4 or more experiments per group.

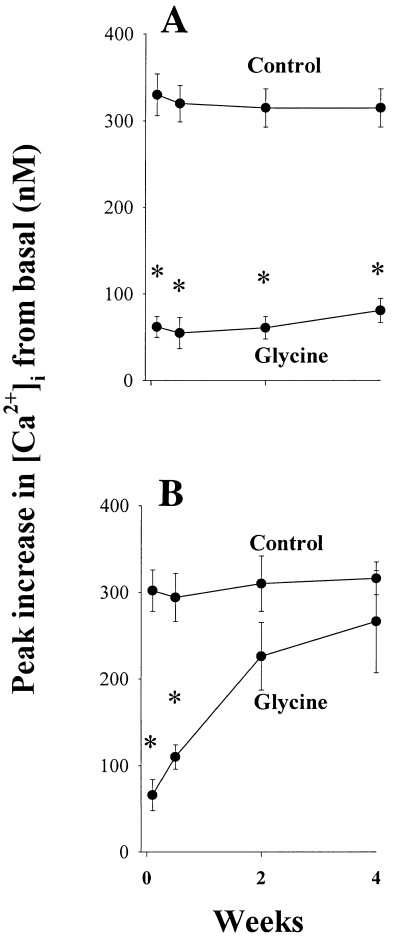

Fig. 5. Time course of downregulation of Kupffer cell glycine-gated chloride channels. Intracellular calcium was measured as described in Fig. 4 in Kupffer cells isolated from Wistar rats fed either casein diet (*A*) or 5% glycine diet (*B*) for 0, 0.5, 2, 4, or 32 wk as described in MATERIALS AND METHODS. Cells were stimulated with 10 μg/ml LPS (control) or incubated in 1 mM glycine for 3 min before stimulation with LPS (glycine). Data are expressed as the peak increase in intracellular calcium concentration ($[Ca^{2+}]_i$) due to LPS and are representative of 4 or more experiments per group (*$P < 0.05$; 2-way ANOVA at each time point; multiple comparison by Tukey's post hoc analysis).

Effect of long-term dietary glycine on alveolar macrophages. Acute respiratory failure and adult respiratory distress syndrome are specific manifestations of endotoxin shock. Alveolar macrophages may contribute to the development of lung injury due to endotoxin shock by releasing toxic mediators such as TNF-α, IL-1, and various free radical species. It has been reported previously that alveolar macrophages can be inactivated in vitro by glycine, most likely through activation of a glycine-gated chloride channel (29). Therefore, it was hypothesized that long-term dietary glycine would prevent LPS-induced mortality by inactivating alveolar macrophages. To test this hypothesis, alveolar macrophages were isolated from animals fed either 5% glycine or casein for 4 wk, and the increase in intracellular calcium due to LPS was assessed. Alveolar macrophages from animals fed casein for 4 wk responded to LPS (10 μg/ml) with a transient increase in intracellular calcium reaching nearly 200 nM and returning to basal levels within 2–3 min (Fig. 6). When glycine (1 mM) was added 3 min before LPS, the increase in $[Ca^{2+}]_i$ was blunted by nearly 90%, confirming earlier work (29). When glycine (1 mM) was added to alveolar macrophages from glycine-fed animals, the LPS-induced increase in $[Ca^{2+}]_i$ was also blunted by about 90%, even after 4 wk of feeding (Fig. 7). These data indicate that the glycine-gated chloride channel on alveolar macrophages, unlike the receptor on Kupffer cells, is not downregulated by increased blood levels of glycine.

Effect of long-term dietary glycine on neutrophil activation. Neutrophils are also involved in lung injury due to endotoxin by infiltrating and adhering in the lung and releasing toxic mediators such as hypochlorous acid, free radicals, and proteases (24). Because the increase in inflammatory cells (i.e., neutrophils) in the lung was largely blocked by glycine after LPS challenge and because it has been reported that neutrophils are inactivated by glycine (28), the possibility that glycine acted directly on the neutrophil was investigated. Neutrophils were isolated from animals fed either control diet or diet containing 5% glycine diet for 4 wk, and the increase in intracellular calcium due to LPS was measured (Fig. 8). Neutrophils from animals

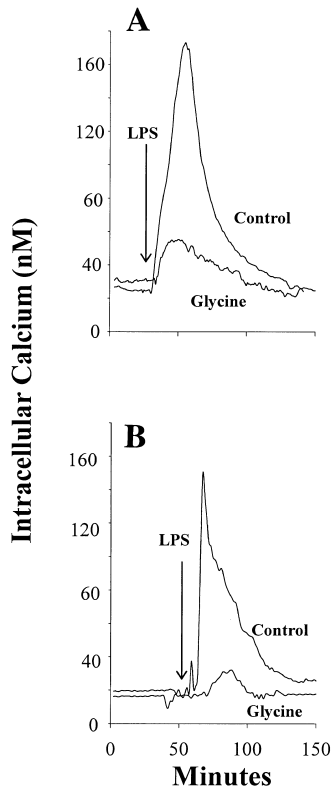

Fig. 6. Glycine inactivates alveolar macrophages after 4 wk of dietary glycine feeding. Intracellular calcium was measured in alveolar macrophages isolated from Wistar rats fed either casein diet (*A*) or 5% glycine diet (*B*) for 4 wk as described in MATERIALS AND METHODS. Cells were incubated in HBSS or HBSS containing glycine (1 mM) for 3 min before addition of 10 μg/ml LPS. Data are representative traces from 4 or more experiments per group.

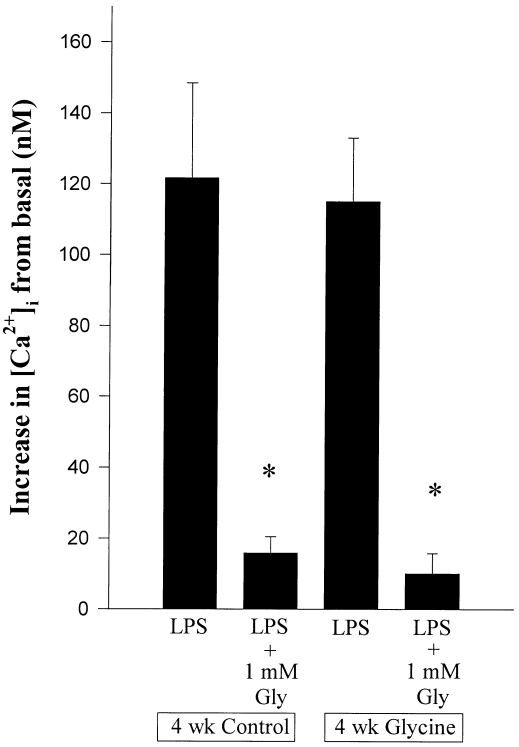

Fig. 7. Effects of long-term dietary glycine (Gly) on alveolar macrophage activation. Intracellular calcium was measured in alveolar macrophages as described in Fig. 4. Cells were stimulated with 10 μg/ml LPS or incubated in 1 mM glycine for 3 min before stimulation with LPS. Data are expressed as the means ± SE of increase in $[Ca^{2+}]_i$ from basal levels for 3 individual experiments ($^*P < 0.01$; 2-way ANOVA; multiple comparison by Tukey's post hoc analysis).

fed casein responded to LPS (100 mg/ml) with a transient increase in intracellular calcium that peaked at 220 ± 20 nM, as expected. Glycine added 3 min before LPS blunted this increase by nearly 80%. Interestingly, the LPS-induced increase in intracellular calcium in neutrophils from animals fed glycine diet was blunted by greater than 60% with 1 mM glycine in vitro. Although the increase in intracellular calcium was significantly blunted in vitro by glycine even after chronic glycine feeding, it was not completely inhibited as in casein-fed controls. Importantly, however, these data indicate that the glycine-gated chloride channel may be minimally downregulated on neutrophils and that neutrophil activation is blunted by glycine even after long-term dietary glycine.

DISCUSSION

Chronic dietary glycine improves survival against endotoxin. Consumption of a glycine-containing diet has been shown to increase survival in an endotoxin model (7). In pharmacological studies with isolated Kupffer cells, glycine blunted LPS-induced increases in intracellular calcium and TNF-α production in a chloride-dependent and strychnine-sensitive manner (7). These data indicate that Kupffer cells are inactivated through glycine-gated chloride channels similar to the inhibitory glycine-gated chloride channels in the nervous system. Moreover, molecular evidence supports the hypothesis that Kupffer cells contain glycine-gated chloride channels similar to the receptor identified in the spinal cord (27). These findings prompted the identification of several other inflammatory cell types that possess glycine-gated chloride channels, including alveolar macrophages (29), neutrophils (28), T cells (20), and splenic macrophages (30). Moreover, short-term dietary glycine has been shown to be protective in several models involving inflammatory cells, such as liver ischemia-reperfusion, primary nonfunction after organ transplantion, and several animal models of cancer (16–18, 34). However, the glycine-gated chloride channel in the spinal cord becomes desensitized following exposure to a high concentration of glycine (1). Accordingly, it was hypothesized that survival following endotoxin shock in animals fed glycine diet for long periods would not be improved if the glycine-gated chloride channel was downregulated. In these studies, the blood glycine concentrations in animals fed 5% glycine were elevated to >1 mM from basal levels of 0.2 mM by feeding (Table 1). In contrast to the hypothesis, animals fed glycine remained tolerant to endotoxin (Fig. 1). Surprisingly, in animals that survived for 24 h after a sublethal dose of LPS, liver injury, measured by serum alanine transaminase and alkaline phosphatase levels, and inflammatory cell influx in glycine-fed ani-

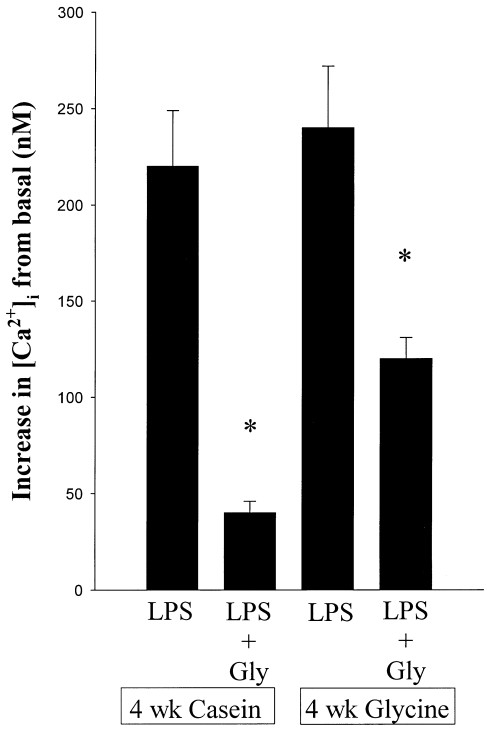

Fig. 8. Effect of long-term glycine diet on LPS-induced neutrophil activation. Intracellular calcium was measured in neutrophils from Wistar rats fed either casein diet or 5% glycine diet for 4 wk as described in MATERIALS AND METHODS. Cells were stimulated with 100 μg/ml LPS or incubated in 1 mM glycine for 3 min before stimulation with LPS. Data are expressed as the means ± SE of increase in $[Ca^{2+}]_i$ from basal levels for 4 or more experiments ($^*P < 0.05$; 2-way ANOVA; multiple comparison by Tukey's post hoc analysis).

mals were not significantly different from animals fed casein for 4 wk (Table 2 and Fig. 2). However, lungs from the glycine-fed animals remained well preserved compared with lungs from control animals, which had increased cellularity of the alveolar wall, lung wet-to-dry weight ratio (Table 2) and inflammatory cell influx (Fig. 2). Most strikingly, the number of infiltrating leukocytes in the lung of glycine-fed animals was reduced by nearly 50% compared with that in the casein-fed control animals. Lymphocyte infiltration and acute lung injury due to endotoxemia have been described (25). In fact, endotoxemia, acute pancreatitis, hemorrhagic shock, and many other disorders have been associated with severe lung inflammation and injury (14, 33). Endotoxin-induced injury is dependent on several inflammatory factors, and specific causes of death have not been established. Thus glycine improves survival by unknown mechanisms, but a reduction of lung inflammation is likely involved.

TNF-α is likely an early mediator of LPS-induced lethality. Because LPS-induced Kupffer cell activation and systemic TNF-α levels are blunted by short-term glycine dietary treatment (6), it was initially hypothesized that Kupffer cells play a large role in the inflammatory response to LPS. However, Kupffer cells are no longer inactivated by glycine after 4 wk of 5% glycine diet, findings inconsistent with the hypothesis that inactivation of Kupffer cells is solely responsible for protection against LPS-induced injury due to glycine (Figs. 4 and 5). Moreover, LPS-induced TNF-α production was not different in glycine-fed animals from casein-fed animals after 4 wk of feeding (Fig. 3), yet rats fed glycine long term had improved survival after endotoxin shock (Fig. 1). Although these data do not exclude TNF-α involvement in lethality, it is unlikely that systemic elevation of TNF-α is solely responsible for mortality due to endotoxin. Most likely, TNF-α plays a role in a complex chain of inflammatory events upsteam from the point where glycine protects. Also, these data are consistent with the hypothesis that glycine may work through other unknown mechanisms, such as inhibiting TNF-α receptor signaling. It is also possible that glycine inhibits TNF-α production from some cells but that other cell types remain insensitive to glycine and produce TNF-α locally in this model. Moreover, it is reasonable to conclude that cell types other than Kupffer cells are responsible for endotoxin-induced mortality.

Alveolar macrophages and circulating neutrophils are inactivated by glycine following long-term dietary glycine. Because LPS-induced Kupffer cell activation and TNF-α levels after LPS administration were not different in glycine- and casein-fed animals after 4 wk of feeding (Fig. 3), the possibility that glycine acts on other cell types (e.g., alveolar macrophages or neutrophils) was considered. The alveolar macrophage is inactivated by glycine and remained sensitive to glycine after long-term glycine feeding (Figs. 6 and 7), indicating that the glycine-gated chloride channel in alveolar macrophages is not downregulated, unlike the receptor in Kupffer cells. A possible explanation for this phenomenon is that the glycine concentration in the alveolar space does not increase with dietary glycine to sufficient levels to downregulate the receptor. Indeed, the glycine concentration in the tracheoalveolar lavage is elevated twofold less than in blood after glycine feeding (Table 1). Moreover, it has been previously reported that neutrophils are inactivated by glycine (28). Here, neutrophils are nearly completely inactivated by glycine even after chronic glycine feeding (Fig. 8), consistent with the hypothesis that glycine-gated chloride channels present in neutrophils are only partially downregulated by glycine. This phenomenon is most likely due to the rapid turnover of circulating neutrophils, which is much more rapid than Kupffer cells.

Role of neutrophil inactivation by glycine in survival against endotoxin. Neutrophils release reactive oxygen species, hypochlorous acid, and many proteolytic enzymes that cause vascular thrombosis, tissue edema, and necrosis (26). In adult respiratory distress syndrome from severe sepsis or pancreatitis, patients with high concentrations of IL-8 and a greater number of neutrophils in the lung had higher rates of mortality (9, 10). In fact, it is argued that infiltrating neutrophils are more critical in lung pathology than tissue macrophages (14, 24). TNF-α is most likely not directly involved in LPS mortality based on the finding that TNF-α levels were not different in casein- and glycine-fed animals after LPS (Fig. 1). However, TNF-α may trigger the recruitment of leukocytes by expression of IL-8 and intracellular adhesion molecules (5, 8). The findings that neutrophils are inactivated by glycine (Fig. 8) in response to endotoxin and neutrophil infiltration in the lung is decreased after endotoxin after 4 wk of consuming a glycine-containing diet are consistent with the hypothesis that neutrophils play a critical role in lung injury due to endotoxin. Whereas neutrophils are stimulated by LPS and glycine blunts activation of neutrophils by LPS and other stimuli (28), neutrophils are more likely activated by a variety of endogenous inflammatory signals (i.e., TNF-α and adhesion molecules). Thus it is reasonable to hypothesize that glycine inactivates neutrophils to a variety of signals that involve intracellular calcium signaling. Here, glycine improves survival by unknown mechanisms, but inactivation of neutrophils and reduction of lung inflammation are likely involved.

It is difficult to link specific tissue damage with cause of death because mortality due to LPS is often the result of systemic shock rather that overt tissue injury. Although these findings do not demonstrate a definitive role for alveolar macrophages and neutrophils in LPS-induced mortality, the data strongly support their involvement in this complex inflammatory cascade. These results provide more insight into the mechanism of LPS-induced lung injury and mortality and are consistent with several reports postulating that neutrophil infiltration is a critical factor in lung injury (14, 24). In conclusion, these data suggest that the long-term consumption of glycine may be beneficial. Other reports have evaluated the effect of acute

consumption of a glycine-containing diet in ischemia-reperfusion models, liver transplantation, arthritis models, and some cancer models (16–18, 30, 34). Thus dietary glycine could be useful for the prevention of lung injury in acute pancreatitis, sepsis, and other inflammatory conditions.

This work was supported by the National Institute on Alcohol Abuse and Alcoholism and Novartis Nutrition.

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