A choline-rich diet improves survival in a rat model of endotoxin shock

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Rivera, Chantal A., Michael D. Wheeler, Nobuyuki Enomo, and Ronald G. Thurman. A choline-rich diet improves survival in a rat model of endotoxin shock. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G862–G867, 1998.—This study investigated whether dietary choline can prevent endotoxin shock. Female Sprague-Dawley rats fed chow or chow plus choline chloride (0.025–0.4%) for 3 days were given lipopolysaccharide (LPS) via the tail vein. Eighty-three percent and 56% of chow-fed rats survived after 2.5 or 5.0 mg/kg LPS, respectively. Choline increased survival in a dose-dependent manner, with maximal effects observed at 0.4%; this dose of choline prevented mortality completely after 2.5 or 5 mg/kg LPS. Choline also improved the microscopic appearance of the lungs and blunted increases in serum aspartate aminotransferase levels. Intracellular Ca$^{2+}$ was monitored in liver and lung macrophages during LPS exposure. Ca$^{2+}$ increases in macrophages from choline-fed rats were blunted by 40–60% compared with chow-fed controls. Feeding choline also blunted tumor necrosis factor-$\alpha$ production. Feeding glycine, which prevents macrophage activation via a chloride channel, in addition to choline was even more effective than feeding choline alone, suggesting that glycine and choline act via distinct sites. These data are consistent with the hypothesis that choline diminishes endotoxin shock by preventing macrophage activation.

Kupffer cells; lipopolysaccharide; tumor necrosis factor-$\alpha$

ENDOTOXIN is a cell wall component of gram-negative bacteria cleared from the systemic circulation largely by Kupffer cells, the resident macrophages of the liver. It has been shown that both circulating and fixed mononuclear phagocytes are activated by endotoxin and release toxic cytokines that mediate injury and mortality observed during exposure to endotoxin. Administration of a lethal dose of endotoxin to rats (endotoxin shock) results in severe hypotension and multiple organ system failure. Liver and lung injury normally appear within 8 h and rats usually die of respiratory failure in 12–24 h. Because mortality is believed to occur due to respiratory failure, it is likely that release of toxic mediators from activated lung macrophages plays a pivotal role in the manifestation of endotoxin shock.

Using a model of intravenous endotoxin administration, Nolan and Ali (13) demonstrated that mortality due to endotoxin shock was increased significantly in rats fed a choline-deficient diet. On the basis of this finding, it was hypothesized that a choline-rich diet may prevent endotoxin shock. The results presented here support this hypothesis and demonstrate that choline blunts macrophage activation due to endotoxin [lipopolysaccharide (LPS)].

METHODS

Dietary treatment. Female Sprague-Dawley rats (250–275 g) were fed standard laboratory chow (Prolab RMH 3000; Agway, Syracuse, NY), chow plus choline chloride (0.025–0.4%), chow plus glycine (5%), or chow plus 5% glycine and 0.4% choline chloride for 3 days. Rats were given free access to water and were maintained on a 12:12-h light-dark cycle. All rats were given adequate care in accordance with institutional guidelines.

Endotoxin treatment. After rats were fed for 3 days on the diets described above, LPS (Escherichia coli serotype O111:B4; Sigma Chemical, St. Louis, MO) suspended in pyrogen-free saline was injected via the tail vein. The dose of LPS ranged from 2.5 to 20 mg/kg. Survival was assessed after 24 h, and in some experiments, blood and tissue samples were collected 8 h after LPS injection. Serum was stored at −20°C for later measurement of aspartate aminotransferase (AST) activity by standard enzymatic methods (1). Lung samples were fixed in phosphate-buffered Formalin and embedded in paraffin. Blind evaluation of hematoxylin and eosin-stained sections was performed.

Kupffer cell isolation. Kupffer cells were isolated from rats fed chow or choline-supplemented diets by collagenase digestion and differential centrifugation as described previously (16). Briefly, the portal vein was cannulated, and livers were perfused with Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution (HBSS; 37°C) for 5 min. Perfusion was continued with HBSS containing 0.025% collagenase type IV (Sigma) for ~5 min. When digestion appeared complete, the liver was removed, placed in a beaker containing collagenase buffer, and cut into small pieces. The suspension was filtered through nylon gauze and centrifuged for 10 min at 450 g at a temperature of 4°C. The cell pellet was resuspended in HBSS, and parenchymal cells were removed by centrifugation at 50 g for 3 min. The nonparenchymal cell fraction was washed twice with buffer. Kupffer cells were isolated by centrifugation through Percoll (Pharmacia, Uppsala, Sweden) at 1,000 g for 35 min. Viability was determined by trypan blue exclusion and was >90%. Cells were seeded on glass coverslips, and culture medium was exchanged after 1 h to remove nonadherent cells. Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin sulfate was used. Purity was determined from the percentage of cells that engulfed latex beads and was found to be near 100%. Cells were cultured for ~24 h before experiments.

Alveolar macrophage isolation. Female Sprague-Dawley rats were fed chow or chow + 0.4% choline for 3 days. Alveolar macrophages were isolated by lavage with sterile PBS via an intratracheal cannula as described elsewhere (2). Cell suspensions were centrifuged at 500 g for 7 min, and red blood cells were lysed with 0.15 M NH$_4$Cl. Cells were resuspended in buffer containing (in mM) 145 NaCl, 5 KCl, 10 HEPES, 5.5 glucose, and 1 mM CaCl$_2$ (pH 7.4). Viability assessed by trypan blue exclusion was >90%.
Measurement of \([\text{Ca}^{2+}]_i\). The fluorescent \(\text{Ca}^{2+}\) indicator dye fura 2 was used to measure intracellular \(\text{Ca}^{2+}\) concentration \(([\text{Ca}^{2+}]_i)\) as detailed previously \((10)\). Briefly, Kupffer cells or alveolar macrophages plated on coverslips were incubated in modified Hanks’ buffer containing 0.03% Pluronic F-127 (BASF; Wyandotte, Wyandotte, MI) at room temperature for 1 h. Changes in fluorescence intensity of fura 2 at excitation (340 and 380 nm) and emission (520 nm) wavelengths were monitored in individual cells, and values were corrected for system noise and autofluorescence as described in detail elsewhere \((10)\).

Tumor necrosis factor-\(\alpha\) measurement. For determination of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) in serum, LPS (10 mg/kg in pyrogen-free saline) was injected via the tail vein. A cannula was implanted in the jugular vein, and blood samples were collected after 0, 15, 30, 45, 60, 90, 120, and 180 min. To each 100-\(\mu\)l blood sample, 30 \(\mu\)l of aprotinin (Sigma) were added. For in vitro TNF-\(\alpha\) measurement, alveolar macrophages were cultured on 24-well plates at a density of \(5 \times 10^5\) cells/well, and medium was exchanged after 1 h to remove nonadherent cells. The culture medium used was DMEM. In some experiments choline was added to the culture medium at a final concentration of 1 mM. After 24 h, the culture medium was replaced with fresh DMEM containing 1 \(\mu\)g/ml LPS, and cells were incubated in the presence or absence of 1 mM choline for an additional 4 h. TNF-\(\alpha\) was measured in serum and culture medium samples by ELISA (Genzyme, Cambridge, MA).

Statistical analysis. Results are means \(\pm\) SE. Significance was determined using Student’s \(t\)-test, Mann-Whitney’s rank-sum test, or Kruskal-Wallis ANOVA on ranks where appropriate. The Fisher’s exact test was used to determine significance in the mortality studies; \(P < 0.05\) was selected before the study as the level of significance.

RESULTS

Effect of dietary choline on survival after LPS injection. There were no significant differences in the average daily consumption of chow or choline-supplemented diets \((18.1 \pm 1.3\) and \(18.5 \pm 1.3\) g, respectively). Body weights for rats in the two groups were also similar \((\text{chow}, 264 \pm 4\) g; \(\text{choline}, 265 \pm 3\) g). After 3 days on the diets, rats received LPS (2.5–20 mg/kg) injected via the tail vein. Of rats fed chow, 17% died within 24 h after 2.5 mg/kg LPS, 56% died at the 5 mg/kg dose, 83% died at the 10 mg/kg dose, and 100% at 20 mg/kg (Fig. 1). Death usually occurred 8–12 h after LPS, and surviving animals showed improvement after 24 h. The addition of 0.4% choline to the diet increased survival to 100% after injection of 2.5 or 5.0 mg/kg LPS \((P < 0.05)\), whereas 44% of rats survived at the 10 mg/kg dose \((Fig. 1)\). To determine the maximal effective dose of choline, the amount of choline added to the diet was varied between 0.025% and 0.4%. Choline improved survival after 5 mg/kg LPS in a dose-dependent manner and was maximally effective at 0.4% \((Fig. 2)\).

Effects of dietary glycine and choline are additive. In previous studies, dietary supplementation with glycine blunted Kupffer cell and alveolar macrophage activation and increased survival due to endotoxin shock \((9, 24)\). Glycine inhibits Kupffer cell activation due to LPS by hyperpolarization of the plasma membrane by activating a glycine-gated \(\text{Cl}^-\) channel \((10)\). To determine if the effects of glycine and choline were additive, rats fed chow, chow supplemented with glycine or choline, or chow supplemented with a combination of glycine and choline were given a lethal dose of LPS (10 mg/kg) as described above. Under these conditions, 17% of chow-

![Fig. 1. Effect of choline on lipopolysaccharide (LPS)-induced mortality. Rats were fed chow or chow supplemented with 0.4% choline for 3 days. LPS was injected into the tail vein at doses indicated, and mortality was assessed after 24 h. Fractions presented are survivors/total. *\(P < 0.05\) (Fisher’s exact test).](http://ajpgi.physiology.org/)

![Fig. 2. Effect of varying dietary choline on survival after LPS. Rats were fed chow with various amounts of added choline chloride for 3 days. LPS (5 mg/kg) was injected via the tail vein, and survival was assessed after 24 h. Fractions represent survivors/total.](http://ajpgi.physiology.org/)
fed and 43% of choline-fed rats survived (Table 1). Glycine alone also improved survival by 50%; however, given together, glycine and choline increased survival to 100%. Since the effects of glycine and choline are additive in this model, it is likely that choline acts at a site distinct from the Cl⁻ channel activated by glycine.

**Effect of dietary choline on serum AST and histology after LPS.** Blood samples were collected 8 h after injection of 5 mg/kg LPS. Basal serum AST values were 65 ± 33 and 55 ± 18 U/l in the chow and chow + 0.4% choline groups, respectively (Fig. 3). Injection of LPS increased AST to 846 ± 146 U/l in chow-fed animals; however, this increase was blunted significantly by feeding choline, with values only reaching 163 ± 22 U/l (P < 0.05). Representative photomicrographs of typical lung samples collected 8 h after treatment of rats with 5 mg/kg LPS are shown in Fig. 4. Increased cellularity, alveolar filling, and inflammation were observed after LPS exposure in rats fed chow (Fig. 4A). Lung pathology was improved markedly by choline (Fig. 4B).

### Table 1. Effect of feeding diets containing glycine and choline on LPS-induced mortality

<table>
<thead>
<tr>
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<th>Survivors/Total</th>
<th>%Survival</th>
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<tbody>
<tr>
<td>No addition</td>
<td>1/6</td>
<td>17</td>
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<tr>
<td>0.4% Choline</td>
<td>3/7</td>
<td>43</td>
</tr>
<tr>
<td>5% Glycine</td>
<td>4/6</td>
<td>67</td>
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<tr>
<td>5% Glycine + 0.4% choline</td>
<td>6/6</td>
<td>100*</td>
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Rats were fed a chow-based diet supplemented with 5% glycine or 0.4% choline chloride. Survival was assessed 24 h after injection of 10 mg/kg lipopolysaccharide (LPS) as described in text (n = 6). * P < 0.05, Fisher’s exact test.

**Effect of dietary choline on changes in [Ca²⁺]i in cultured macrophages.** Increased [Ca²⁺]i is an essential precursor event for the production of proinflammatory cytokines (17) and was used here as a marker of Kupffer cell and alveolar macrophage activation (Figs. 5A and 6A). Intracellular [Ca²⁺]i was monitored fluorometrically in individual cells isolated from chow- or choline-fed rats. After the addition of 10 µg/ml LPS to the culture medium of Kupffer cells isolated from chow-fed rats, [Ca²⁺]i increased rapidly, reaching a peak value of 241 ± 16 nM within 100 s, followed by a decline to basal levels within 200 s (Fig. 5A). However, when Kupffer cells were isolated from rats fed 0.4% choline for 3 days, the increase in [Ca²⁺]i due to LPS was only ~60% as large as the response in Kupffer cells isolated from chow-fed rats (Fig. 5B). Similarly, LPS-

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**Fig. 3.** Effect of choline on LPS-stimulated serum aspartate aminotransferase (AST) levels. Conditions are as described in Fig. 1 legend. Blood samples were collected 8 h after injection of 5 mg/kg LPS. * P < 0.05 compared with control. # P < 0.05 compared with chow + LPS (Kruskal-Wallis ANOVA on ranks; n = 4).

**Fig. 4.** Effect of choline on lung histology after LPS injection. Conditions are as described in Fig. 1 legend. Representative lung specimens were collected for histology 8 h after LPS injection (5 mg/kg) and stained with hematoxylin and eosin. A: chow-fed rat. B: chow + 0.4% choline-fed rat. Original magnification, ×40.
stimulated \( [\text{Ca}^{2+}]_i \) reached peak values of 207 \( \pm \) 6 nM in alveolar macrophages from chow-fed rats (Fig. 6A).

\[ \text{[Ca}^{2+}]_i \text{ in alveolar macrophages isolated from rats fed a choline-rich diet only reached 72} \pm \text{7 nM and was significantly lower than the response observed in cells from rats fed chow (Fig. 6, A and B). The addition of 10 mM choline chloride to the culture medium 6 or 24 h before LPS did not alter increases in Ca}^{2+} \text{ caused by LPS (data not shown).} \]

Effect of dietary choline on TNF-\( \alpha \) production. Serum TNF-\( \alpha \) was measured at various times after injection of 10 mg/kg LPS. In chow-fed rats, serum TNF-\( \alpha \) began to increase 45 min after LPS and reached peak values of 5,369 \( \pm \) 1,378 pg/ml at 90 min. Choline had no effect on the increase in serum TNF-\( \alpha \) due to LPS, with peak values reaching 4,475 \( \pm \) 967 pg/ml at 90 min. To determine the effect of choline on alveolar macrophages, cells were isolated from chow- or choline-treated rats and exposed to LPS in the culture medium (Fig. 6C). Alveolar macrophages isolated from chow-fed rats produced TNF-\( \alpha \) at a rate of 1,018 \( \pm \) 190 pg \( \cdot \) 5 \( \times \) 10^2 cells \( \cdot \) h^{-1}. Feeding rats a choline-rich diet before cell isolation significantly diminished TNF-\( \alpha \) produc-

Fig. 5. Effect of choline on LPS-stimulated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in isolated Kupffer cells. [Ca\(^{2+}\)]\(_i\) levels in cultured Kupffer cells from chow- or chow + choline-fed rats were measured fluorometrically using fura 2 as described in METHODS. Addition of LPS (10 \( \mu \)g/ml) is denoted with arrows. A: representative traces. B: mean \( \pm \) SE of peak [Ca\(^{2+}\)]\(_i\) following LPS. *** \( P < 0.001 \), Student’s t-test; \( n = 4 \) rats/group.

Fig. 6. Effect of choline on alveolar macrophages. Alveolar macrophages were isolated from chow- or choline-fed rats. A: representative traces of [Ca\(^{2+}\)]\(_i\) measured fluorometrically as described in METHODS. B: average peak increase in [Ca\(^{2+}\)]. * \( P < 0.05 \), Mann-Whitney’s rank-sum test. C: alveolar macrophages were cultured in presence of 1 \( \mu \)g/ml LPS as detailed in METHODS. Tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) was measured by ELISA. * \( P < 0.05 \) using Kruskal-Wallis ANOVA on ranks.
tion (543 ± 158 pg·5 × 10⁵ cells⁻¹·4 h⁻¹); however, LPS-stimulated production of TNF-α was not diminished when choline was added in vitro to the culture medium of cells isolated from chow-fed rats (1,200 ± 130 pg·5 × 10⁵ cells⁻¹·4 h⁻¹).

**DISCUSSION**

Dietary choline reduces tissue injury and mortality in the rat. Because choline deficiency enhances injury and mortality due to endotoxin shock (13), it was hypothesized that dietary supplementation with choline would diminish these effects. Standard chow diet contains ~0.12% choline. In the present study, rats were fed diets supplemented with 0.4% choline for 3 days before endotoxin was given. The addition of choline prevented mortality completely at a dose of LPS sufficient to cause death in 50% of rats fed standard chow diet (Fig. 1). The protective effect of choline against mortality was associated with a marked reduction in lung injury (Fig. 5). Because mortality due to endotoxin shock is believed to result from respiratory failure in this model, these data are consistent with the hypothesis that choline improves survival by preventing lung pathology.

How does choline prevent mortality? Previously, it has been shown (9) that dietary supplementation with glycine blocks endotoxin-induced injury and subsequent mortality. The protective effect of glycine was associated with smaller LPS-induced increases in [Ca²⁺], in isolated Kupffer cells and alveolar macrophages as well as with a decrease in TNF-α production (1, 24), a Ca²⁺-dependent event (22). Glycine blunted activation when added to the diet or when added directly to the culture medium of isolated cells by stimulating the influx of Cl⁻, thus hyperpolarizing the cell membrane (10). As described in this study, feeding a diet rich in both glycine and choline was even more effective in preventing mortality than feeding choline or glycine alone (Table 1). In contrast to glycine, in vitro treatment of macrophages with choline did not prevent LPS stimulation of [Ca²⁺], or TNF-α. These findings suggest that the protective mechanism against endotoxin shock observed after dietary supplementation with choline is distinct from glycine.

Dietary choline inhibits macrophage activation. A previous study (8) demonstrated that macrophage activation by LPS was associated with an increased rate of phosphatidylcholine hydrolysis via a phospholipase-dependent mechanism. Hydrolysis of phosphatidylcholine by phospholipase C and D generates 1,2-diacylglycerol (DAG) (3). Recent experiments (26) have shown that DAG formation and protein kinase C-ζ activation correlate with the activation of nuclear factor-κB, a transcription factor necessary for production of many cytokines, including TNF-α. Cytokines contribute to injury and mortality observed during LPS exposure (20). For example, circulating levels of TNF-α and interleukin-1 (IL-1) increase rapidly after LPS exposure (4). Treatment of rats with TNF-α mimics the changes in lipid metabolism and mortality caused by LPS (12, 18), whereas antibodies directed against TNF-α prevent these effects (6, 19). Although administration of IL-1 can reproduce many of the changes in lipid metabolism associated with LPS exposure, antibodies directed against IL-1 were not effective (7, 12). Therefore, it is likely that production of TNF-α is of primary importance in the mechanism of endotoxin shock.

One possible explanation for the findings presented here is that excess choline supplied in the diet could...
increase the ratio of phosphatidylcholine to polyphosphoinositides in the cell membrane (8). This could prevent signaling events downstream of LPS binding to receptors on macrophages, since phosphoinositides, but not phosphatidylcholine, lead to increases in Ca^{2+} necessary for macrophage activation (3). Alternatively, LPS decreases membrane fluidity, an effect reversed by the addition of phosphatidylcholine (11). Supplying excess choline in the diet could alter intracellular signaling due to LPS by enhancing phosphatidylcholine resynthesis and maintaining proper membrane fluidity (see Fig. 7). Data presented here demonstrate that the LPS-stimulated increase in intracellular Ca^{2+} was blunted by about 50% in Kupffer cells and alveolar macrophages isolated from choline-fed rats (Figs. 5 and 6). Furthermore, TNF-α production by alveolar macrophages was diminished ~50% by choline (Fig. 6C). Although the exact mechanism underlying the protective effects of choline is not yet clear, the findings reported here strongly support the hypothesis that choline interferes with the intracellular signaling cascade triggered by LPS.

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