Production of superoxide and TNF-α from alveolar macrophages is blunted by glycine

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Wheeler, Michael D., and Ronald G. Thurman. Production of superoxide and TNF-α from alveolar macrophages is blunted by glycine. *Am. J. Physiol.* 277 (*Lung Cell. Mol. Physiol.* 21): L952–L959, 1999.—Glycine blunts lipopolysaccharide (LPS)-induced increases in intracellular calcium concentration ($[Ca^{2+}]_i$) and tumor necrosis factor-α (TNF-α) production by Kupffer cells through a glycine-gated chloride channel. Alveolar macrophages, which have a similar origin as Kupffer cells, play a significant role in the pathogenesis of several lung diseases including asthma, endotoxemia, and acute inflammation due to inhaled bacterial particles and dusts. Therefore, studies were designed here to test the hypothesis that alveolar macrophages could be inactivated by glycine via a glycine-gated chloride channel. The ability of glycine to prevent endotoxin [lipopolysaccharide (LPS)]-induced increases in $[Ca^{2+}]_i$ and subsequent production of superoxide and TNF-α in alveolar macrophages was examined. LPS caused a transient increase in intracellular calcium to nearly 200 nM, with EC_{50} values slightly greater than 25 ng/ml. Glycine, in a dose-dependent manner, blunted the increase in $[Ca^{2+}]_i$, with an IC_{50} less than 100 μM. Like the glycine-gated chloride channel in the central nervous system, the effects of glycine on $[Ca^{2+}]_i$ were both strychnine sensitive and chloride dependent. Glycine also caused a dose-dependent influx of radiolabeled chloride with EC_{50} values near 10 μM, a phenomenon which was also inhibited by strychnine (1 μM). LPS-induced superoxide production was also blunted in a dose-dependent manner by glycine and was reduced ~50% with 10 μM glycine. Moreover, TNF-α production was also inhibited by glycine and also required nearly 10 μM glycine for half-inhibition. These data provide strong pharmacological evidence that alveolar macrophages contain glycine-gated chloride channels and that their activation is protective against the LPS-induced increase in $[Ca^{2+}]_i$ and subsequent production of toxic radicals and cytokines.

glycine-gated chloride channel; intracellular calcium; tumor necrosis factor-α

ALVEOLAR MACROPHAGES ARE the lung's central defense against inhaled particles and pathogens. In the phagocytosis of foreign particles, the macrophage becomes activated and releases many toxic mediators such as tumor necrosis factor-α (TNF-α), superoxide ($O_2^-\cdot$), nitric oxide, granulocyte-macrophage colony-stimulating factor, and other inflammatory cytokines (2, 3). In particular, the inhalation of organic or cotton dusts, which are highly contaminated with bacterial endotoxin [lipopolysaccharide (LPS)], results in the overproduction and release of radicals and TNF-α largely from alveolar macrophages (33). Moreover, LPS in septic patients stimulates alveolar macrophages to release cytokines, particularly TNF-α (11), which lead to adult respiratory distress syndrome (ARDS), a disease associated with a high rate of mortality (26, 29, 30). Accumulating evidence also indicates a role for macrophages in the pathogenesis of both hypersensitivity reactions and bronchial asthma (8, 18). The macrophage releases cytokines that induce histamine release from basophils and enhance the inflammatory potential of eosinophils (12, 17).

LPS-induced macrophage activation is typically characterized by an initial increase in intracellular calcium concentration ($[Ca^{2+}]_i$), which is necessary for the production of superoxide and TNF-α and the induction of several inflammatory responses (6). LPS activates macrophages, in part, via the cell surface receptor CD14, which leads to the release of calcium from the endoplasmic reticulum through inositol trisphosphate-dependent channels (6, 31). Furthermore, $[Ca^{2+}]_i$ is increased by calcium influx through voltage-dependent calcium channels, which are activated by membrane depolarization (13). The increase in $[Ca^{2+}]_i$ is involved in the signaling of macrophage production of TNF-α and free radicals, most likely by activating calcium-dependent kinases (10, 14).

Glycine, a nonessential amino acid, has been shown to be protective against hypoxia, ischemia, and various cytotoxic substances in renal proximal tubules via glycine-gated chloride channels (23, 35). Recently, it was reported that dietary glycine prevents liver and lung injury due to lethal doses of LPS in the rat (15). It has since been shown that glycine blunts the transient increases in $[Ca^{2+}]_i$ and production of TNF-α in response to LPS in the resident hepatic macrophage, the Kupffer cell (16). It was concluded that Kupffer cells contain a glycine-gated chloride channel that hyperpolarizes the plasma membrane, making voltage-dependent calcium influx more difficult, thus preventing activation of the macrophage (16).

Because alveolar macrophages are critically involved in the pathogenesis of many pulmonary diseases caused by inhaled particles and endotoxins, these studies were designed to test the hypothesis that alveolar macrophages could be inactivated by glycine via a glycine-gated chloride channel. Alveolar macrophages are important because of their diverse role of phagocytosis of exogenous particles such as cotton dust. The release of inflammatory cytokines and toxic free radicals by alveolar macrophages stimulated by LPS is dependent on increases in $[Ca^{2+}]_i$ (10, 14). Therefore, the ability of glycine to prevent LPS-induced increases in $[Ca^{2+}]_i$ and to modulate the subsequent production of $O_2^-\cdot$ and

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TNF-α was also examined. Preliminary accounts of this work have appeared elsewhere (36).

METHODS

Isolation of alveolar macrophages. Alveolar macrophages were isolated from Sprague-Dawley rats (300–350 g) by bronchoalveolar lavage. Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg body wt). The lungs were lavaged eight times with 8-ml aliquots of PBS (145 mM NaCl, 1.9 mM NaH_2PO_4, and 9.35 mM Na_2HPO_4, pH 7.4) via a cannula inserted into the trachea. Lavage suspensions were centrifuged at 500 *g* for 7 min at 4°C. Red blood cells were lysed with 0.15 M NH_4Cl, and cells were suspended in HEPES-buffered medium (in mM: 145 NaCl, 5 KCl, 10 HEPES, 5.5 glucose, and 1 $CaCl_2$, pH 7.4). Cell viability determined by trypan blue exclusion was >94%.

Cells were resuspended in DMEM (4,500 mg/l glucose) supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) and were plated at the desired density and incubated for 1 h at 37°C. Nonadherent cells were removed by replacing medium with fresh DMEM. Adherent cells were cultured at 37°C with 5% CO_2 for 24–48 h before experiments.

Measurement of $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ of single cells were measured fluorometrically using the calcium indicator fura 2 (16). Briefly, cells were plated on glass coverslips at a density of 3.0×10^5 cells/coverslip and were incubated in 2 ml modified HBSS (m-HBSS) (in mM: 15 HEPES, 110 NaCl, 5 KCl, 0.3 Na_2HPO4, 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-AM (Molecular Probes, Eugene, OR) at room temperature for 30 min. After loading, cells were rinsed and placed in a measurement chamber with m-HBSS buffer at room temperature. A microspectrofluorometer (Photon Technology International, South Brunswick, NJ) attached to an inverted microscope (Diaphot, Nikon) was used to monitor changes in $[Ca^{2+}]_i$. 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 alveolar macrophages. The ratio of emission at 340 to 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.

Measurement of radiolabeled chloride influx. To determine if glycine could stimulate influx of extracellular chloride into alveolar macrophages, a radiolabeled chloride flux assay described by Morrow and Paul (25) was used. Briefly, 5×10^5 cells were plated on coverslips in 60-cm^2 culture dishes in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate). Cells were incubated at 37°C in a 5% CO_2-containing atmosphere for 24 h and washed with HEPES buffer (in mM: 20 HEPES, pH 7.4, 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, and 2.5 $CaCl_2$) before the assay. Glycine (1 μM to 1 mM) was diluted in HEPES buffer and was added to 60-cm^2 petri dishes. Radiolabeled chloride (^{36}Cl final concentration 2 μCi/ml) was added to the glycine solution and kept at room temperature. Coverslips with adherent cells were then incubated in chloride solution at room temperature for 5 s, removed rapidly into ice-cold wash buffer for 3 s, and then transferred into a second ice-cold wash buffer for 7 s. Coverslips were broken, transferred to scintillation vials containing 1.6 ml of 0.2 N NaOH, and incubated for 1 h. A 160-μl aliquot was taken for protein quantification by the Lowry method (20). The sample was then diluted in 10 ml scintillation fluid, and radioactivity was counted.

Measurement of $O_2^-\cdot$ production. Alveolar macrophage $O_2^-\cdot$ production was measured by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* (22). Cells were plated in 24-well tissue culture plates at 10^6 cells/well and cultured at 37°C for 24 h in DMEM with 10% FBS. Supernatant was replaced with m-HBSS and Ca^{2+} supplemented with ferricytochrome *c* (0.8 mg/ml final concentration). Glycine (0.1 μM to 1 mM) was added 5 min before LPS. The reduction of ferricytochrome *c* was measured both in the presence and absence of SOD (85 U/ml). The difference in absorbance of ferricytochrome *c*, measured at 550 nm, was used to calculate $O_2^-\cdot$ concentration using a molar extinction coefficient of 17,500.

Measurement of TNF-α release in culture media. Isolated alveolar macrophages were cultured in 24-well culture plates at a density of 0.5×10^6 cells/well in glycine-free DMEM for 24 h, as described by Ikejima et al. (16). Cells were then incubated with LPS (1–1,000 ng/ml) in the presence or absence of glycine (1 μM to 1 mM) at 37°C for 4 h. TNF-α in the culture medium was determined using an ELISA kit (Genzyme, Cambridge, MA).

RESULTS

Glycine blocks LPS-induced increases in $[Ca^{2+}]_i$ in alveolar macrophages. The $[Ca^{2+}]_i$ in individual alveolar macrophages was determined fluorometrically with the calcium indicator fura 2 as described in METHODS. The addition of LPS plus 5% rat serum caused a transient increase in $[Ca^{2+}]_i$ that reached maximal levels within 60 s and rapidly returned to basal levels (i.e., ~10–50 nM) within 3–5 min (Fig. 1). LPS (plus 5% rat serum) induced increases in $[Ca^{2+}]_i$ to a peak concentration of nearly 200 nM in a dose-dependent manner (half-maximal effect with 26 ng/ml LPS) (Fig. 2). The addition of 5% rat serum provided the LPS-binding protein (LBP) that enhanced the response of the alveolar macrophage, making the cell more sensitive to LPS by nearly 100-fold, confirming work by others (data not shown) (21).

Glycine (1 mM) added 3 min before stimulation with LPS nearly completely prevented the increase in $[Ca^{2+}]_i$ due to LPS, with values only increasing to 15 nM (Fig. 3). Modulation of LPS-induced increases in $[Ca^{2+}]_i$ by glycine was also shown to be dose dependent, with an IC_{50} observed with nearly 10 μM glycine (Fig. 3). Glycine alone had no measurable effect on $[Ca^{2+}]_i$ (data not shown).

Strychnine antagonizes inhibition of LPS-induced increases in $[Ca^{2+}]_i$ by glycine. The glycine-gated chloride channel that has been characterized in the spinal cord is known to be inhibited by low concentrations of strychnine. Therefore, to test the hypothesis that alveolar macrophages contain a strychnine-sensitive glycine-gated chloride channel, strychnine was added 3 min before glycine. Strychnine (1 μM) largely prevented the

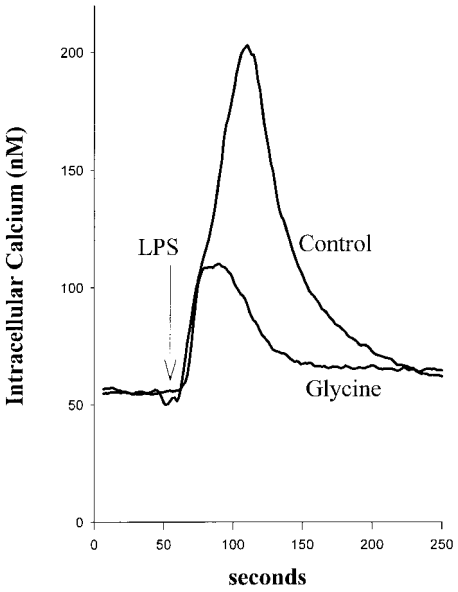

Fig. 1. Effects of lipopolysaccharide (LPS) and glycine on intracellular calcium concentration ($[Ca^{2+}]_i$) in isolated alveolar macrophages. $[Ca^{2+}]_i$ was measured on an individual cell fluorometrically using fluorescent calcium indicator fura 2 as described in METHODS. LPS (1 μg/ml) plus 5% rat serum was added in modified HBSS (m-HBSS), and glycine (1 mM) was added 3 min earlier. Data are representative of experiments performed on cells isolated from 4–6 individual animals.

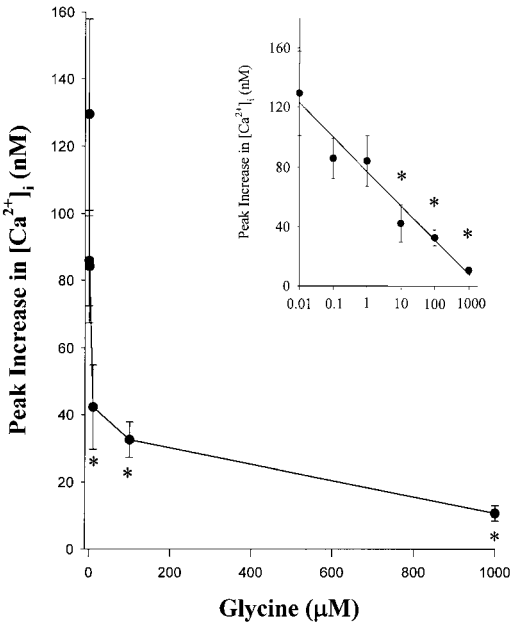

Fig. 3. Dose-response curve for glycine on LPS-induced peak $[Ca^{2+}]_i$. Experimental conditions are as described in Fig. 1. Alveolar macrophages were incubated in glycine-containing m-HBSS for 3 min before addition of LPS (1 μg/ml). Data are represented as peak $[Ca^{2+}]_i$ above basal $[Ca^{2+}]_i$ for each individual cell. *Inset*: data plotted on a logarithmic scale to demonstrate linearity. Data are expressed as means ± SD of experiments from cells isolated from 3–6 individual animals (linear regression: $*P < 0.05$, ANOVA with Tukey's post hoc analysis for comparison with control).

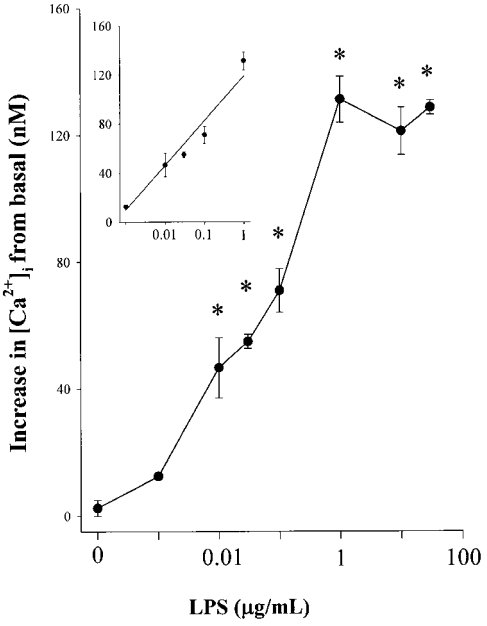

Fig. 2. Dose dependence of LPS on increase in $[Ca^{2+}]_i$ in alveolar macrophages. $[Ca^{2+}]_i$ was measured on an individual cell fluorometrically using fluorescent calcium indicator fura 2 as described in METHODS. LPS plus 5% rat serum in m-HBSS was added to isolated alveolar macrophages. Rat serum alone yielded no response and was used as control. *Inset*: data plotted on a logarithmic scale to demonstrate linearity. Data are represented as peak $[Ca^{2+}]_i$ above basal concentrations and are expressed as means ± SD of experiments performed on cells isolated from 4–6 individual animals (linear regression: $*P < 0.05$, ANOVA with Tukey's post hoc analysis for comparison with control).

inhibitory effects of glycine (1 mM) on LPS-induced increases in $[Ca^{2+}]_i$ (Fig. 4). The peak $[Ca^{2+}]_i$ values due to LPS in the presence of glycine (1 mM) were not significantly different from control levels in the presence of strychnine (1 μM). Strychnine (1 μM) alone also had no effect on $[Ca^{2+}]_i$ levels (data not shown).

As a partial agonist to the glycine-gated chloride channel, high concentrations of strychnine mimic the effects of glycine in several models (24). High-dose strychnine (1 mM) added 3 min before the LPS stimulation totally inhibited the increase in $[Ca^{2+}]_i$ in alveolar macrophages due to LPS like glycine (Fig. 4).

Inhibition of LPS-induced increases in $[Ca^{2+}]_i$ by glycine is dependent on extracellular chloride. Activation of the glycine-gated chloride channel allows the influx of chloride ions that hyperpolarize the cell membrane, thus preventing increases in $[Ca^{2+}]_i$ via voltage-dependent channels (32). Therefore, replacing sodium chloride with sodium gluconate in the extracellular assay buffer should prevent glycine from blunting LPS-induced increases in $[Ca^{2+}]_i$. Indeed, glycine (1 mM) in normal chloride-containing buffer nearly completely inhibited the increase of $[Ca^{2+}]_i$ due to LPS; however, in chloride-free buffer, glycine had no effect (Fig. 5). LPS could still increase $[Ca^{2+}]_i$ in the absence of extracellular chloride to peak levels similar to those observed in the presence of chloride, suggesting that extracellular chloride depletion does not directly influence the LPS-induced increase in $[Ca^{2+}]_i$. Therefore, it is concluded that the inhibitory effects observed by glycine are dependent on the presence of chloride,

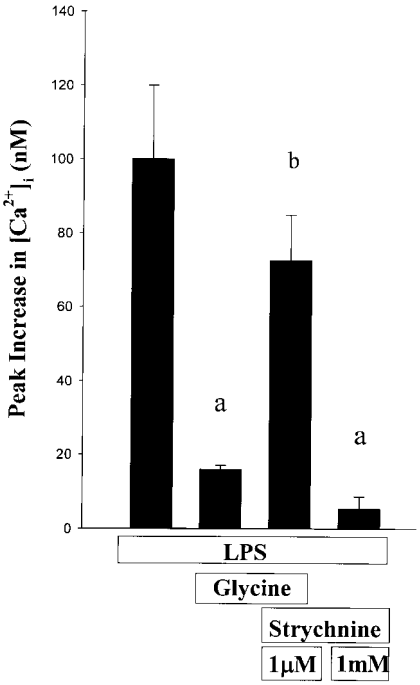

Fig. 4. Effects of strychnine on LPS-induced increases in $[Ca^{2+}]_i$. Experimental conditions are as described in Fig. 1. Strychnine (1 μM) was added to buffer 3 min before glycine (1 mM). After incubation with strychnine and glycine for 3 min, LPS (1 μg/ml plus 5% serum) was added. In experiment using a high concentration of strychnine in absence of glycine, strychnine (1 mM) was added to buffer 3 min before LPS. Data are expressed as peak $[Ca^{2+}]_i$ above basal $[Ca^{2+}]_i$ for each individual cell. Data are expressed as means ± SD of experiments performed on cells isolated from 3 or 4 individual animals ($^aP < 0.05$ compared with LPS-treated controls, $^bP < 0.05$ compared with LPS-treated cells in presence of glycine; 2-way ANOVA with Tukey's post hoc analysis for comparison with LPS-treated control).

consistent with the hypothesis that glycine acts via a glycine-gated chloride channel.

Glycine stimulates influx of radiolabeled chloride. The glycine-gated chloride channel promotes the influx of chloride into the cell that is hypothesized to hyperpolarize the plasma membrane, preventing LPS-induced increases in $[Ca^{2+}]_i$. Although the pharmacological data above support the hypothesis that glycine acts through a glycine-gated chloride channel, influx of radiolabeled chloride into the cells stimulated by glycine would provide hard physical evidence that chloride movement across the membrane is stimulated by glycine and would strongly support the presence of a glycine-gated chloride channel in alveolar macrophages. Indeed, radiolabeled chloride influx was stimulated with glycine in a dose-dependent manner, with an EC_{50} of ~10 μM. Glycine (0.1 mM) caused a significant 2.5-fold influx of radiolabeled chloride (Fig. 6*A*). Furthermore, the effect of glycine was blocked by 1 μM strychnine (Fig. 6*B*). These data provide strong evidence for the presence of a glycine-sensitive chloride channel in alveolar macrophages.

Glycine blocks the LPS-induced production of $O_2^-\cdot$. The production of superoxide by alveolar macrophages was measured from the SOD-inhibitable reduction of ferricytochrome *c* to its ferrous form. LPS (1 μg/ml plus 5% serum) increased $O_2^-\cdot$ production to 12.4 nmol·10^6 cells^{-1}·30 min^{-1} from basal values of 2.2 nmol·10^6 cells^{-1}·30 min^{-1} (Fig. 7). LPS-induced $O_2^-\cdot$ production was inhibited by glycine in a dose-dependent manner with an IC_{50} of 1 μM and was blocked completely by 1 mM glycine.

Glycine blocks the LPS-induced release of TNF-α by alveolar macrophages. To evaluate the effects of glycine on cytokine release from alveolar macrophages, LPS-induced TNF-α release was measured using an ELISA. As expected, LPS (1 ng/ml to 1 μg/ml) increased TNF-α production, with an EC_{50} of 10–30 ng/ml (Fig. 8*A*), consistent with the EC_{50} for LPS-induced increases in $[Ca^{2+}]_i$ (Fig. 1). Moreover, glycine blunted the LPS-induced release of TNF-α significantly in a dose-dependent manner, with an IC_{50} value near 10 μM (Fig. 8*B*). This concentration of glycine also caused a 50% decrease in the LPS-induced peak increase in $[Ca^{2+}]_i$ (Fig. 3).

DISCUSSION

Glycine blunts increases in $[Ca^{2+}]_i$ in alveolar macrophages. There are many disease states in which the response of macrophages to LPS leads to the overproduction of toxic mediators such as cytokines and free radicals. In particular, alveolar macrophages respond to LPS to produce inflammatory mediators such as TNF-α and superoxide that are implicated in respiratory conditions in septic shock and ARDS. Although the

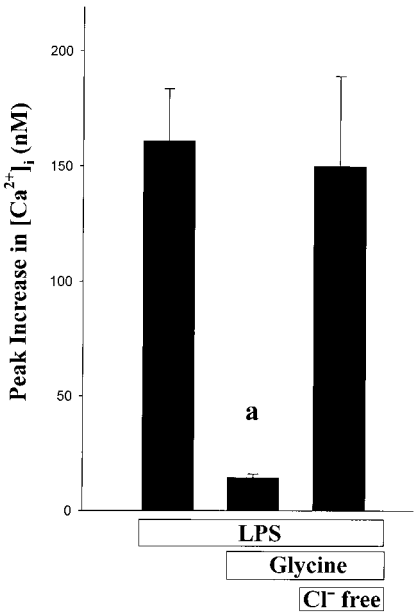

Fig. 5. Effects of glycine on LPS-induced increases in $[Ca^{2+}]_i$ in chloride-free buffer. Experimental conditions are as described in Fig. 1. Alveolar macrophages were incubated in chloride-free buffer by substitution of sodium chloride with sodium gluconate for 3 min before addition of glycine (1 mM). After macrophages were incubated in chloride-free buffer containing glycine for 3 min, LPS (0.1 μg/ml plus 5% serum) was added. Data are represented as peak $[Ca^{2+}]_i$ above basal $[Ca^{2+}]_i$ for each individual cell and expressed as means ± SD of experiments performed on cells isolated from 3 or 4 individual animals ($^aP < 0.01$ compared with LPS-treated control, ANOVA with Tukey's post hoc comparison).

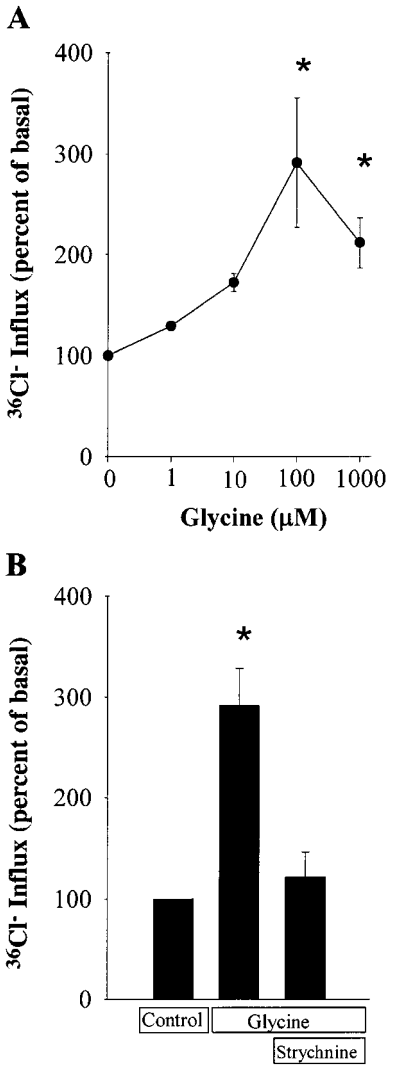

Fig. 6. Glycine promotes influx of radiolabeled chloride. Cells (5.0×10^5) were plated onto 25-mm^2 coverslips and incubated for 24 h at 37°C in DMEM supplemented with 10% FBS and antibiotics. Cells were washed with HEPES buffer before experiments described in METHODS. *A*: coverslips were added to buffer containing radiolabeled chloride and increasing concentrations of glycine for 5 s and washed twice in cold buffer. *B*: cells were added to buffer containing radiolabeled chloride alone, buffer containing radiolabeled chloride and 0.1 mM glycine, or buffer containing radiolabeled chloride, 0.1 mM glycine, and 1 μM strychnine for 5 s, then washed twice in cold buffer. Counts were normalized to amount of protein on each coverslip. Data are representative of at least 4 experiments done in triplicate and are expressed as percent of control in each experiment (linear regression: $*P < 0.05$, ANOVA with Tukey's post hoc comparison).

exact mechanisms that lead to mortality are uncertain, it is thought that these mediators are involved (26, 29, 30).

Recent reports have suggested that alveolar macrophages may be much more sensitive to LPS than other macrophage/monocytes such as the peritoneal macrophage and the Kupffer cell, the resident hepatic macrophage (27). In this study, LPS caused a transient increase in calcium and a rapid return to basal levels (~15–60 nM) within minutes. Moreover, the EC_{50} for LPS on the increase in $[Ca^{2+}]_i$ was 26 ng/ml, a concentration which is consistent with the literature (21). However, peak increases in $[Ca^{2+}]_i$ due to LPS in alveolar macrophages occurred in response to 1.0 μg/ml LPS in the presence of 5% rat serum (Fig. 2) as opposed to higher values needed in other cell types (16). The differences in responsiveness observed in these cells compared with other macrophages may be related to their exposure to LPS. Kupffer cells are continuously exposed to low circulating levels of LPS and may become desensitized over time. Alveolar macrophages, present in surfactant lining of the lung, are limited in their exposure to bloodborne LPS and thus may remain highly sensitive to airborne pathogens. Because LPS binds the LBP and interacts with the cell surface receptor CD14 expressed on a variety of cell types, differences in sensitivity may also be related to differences in the expression levels of receptor (21). This idea is supported by two lines of work. First, exposure to acute ethanol in vivo decreased responsiveness to LPS-induced production of TNF-α in isolated hepatic macrophages (7). Second, this effect was inhibited by reducing gut endotoxin with antibiotic treatment, suggesting that acute ethanol increases gut-derived LPS, which desensitizes macrophages against subsequent LPS stimulation (1). Other receptors are also expressed on macrophages that bind LPS and elicit inflammatory responses as well. Some of these include the scavenger receptors (SR-A and SR-B) and β-integrins, and the participation of these receptors in the LPS response cannot be excluded in this model (9). In fact, it is highly likely that all of these signaling pathways are to some extent involved.

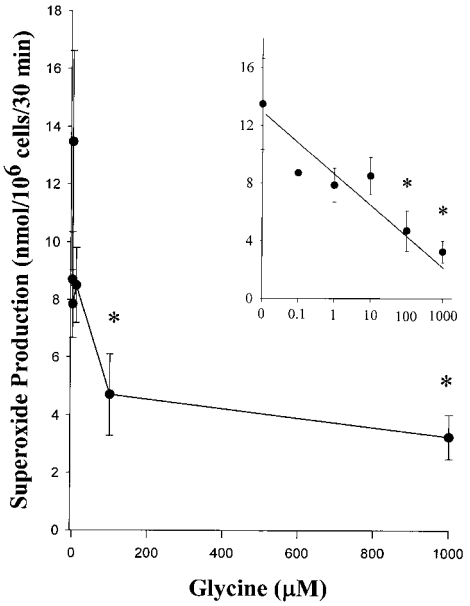

Fig. 7. Effect of glycine on LPS-induced superoxide production. Superoxide production was measured by superoxide dismutase-inhibitable reduction of ferricytochrome *c* as described in METHODS. Cells were incubated in either presence or absence of glycine 3 min before addition of LPS (1 μg/ml plus 5% serum). *Inset*: data are plotted on a logarithmic scale to demonstrate linearity. Data are expressed as means ± SD and are representative of 3 individual experiments (linear regression: $*P < 0.05$, ANOVA with Tukey's post hoc analysis for comparison with control).

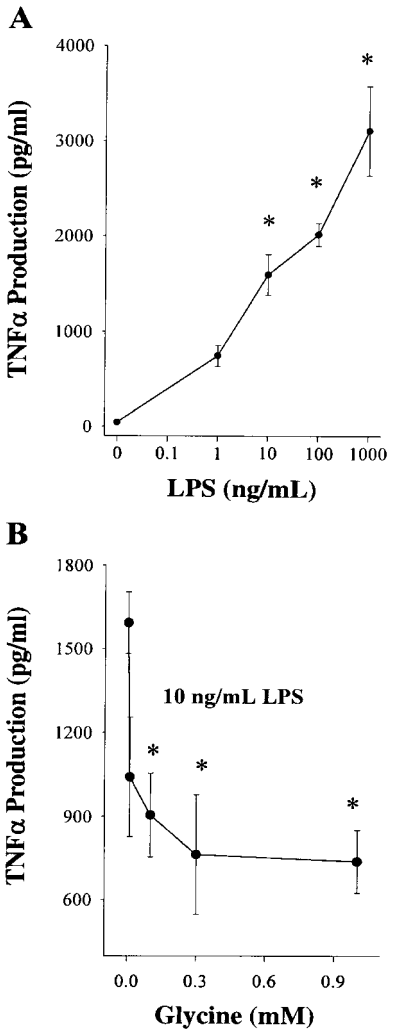

Fig. 8. Effect of glycine on LPS-induced tumor necrosis factor-α (TNF-α) release. TNF-α from isolated alveolar macrophages was measured in culture medium by ELISA (Genzyme). *A*: alveolar macrophages were cultured in glycine-free DMEM plus LPS (1–1,000 ng/ml) plus 5% rat serum for 4 h. *B*: alveolar macrophages were cultured in glycine-free DMEM (controls) and DMEM supplemented with glycine (1 μM to 1 mM) and exposed to LPS (10 ng/ml) for 4 h. Data are representative of 3–5 individual experiments and are expressed as percent of control (linear regression: $*P < 0.05$, ANOVA with Tukey's post hoc analysis for comparison with glycine-free controls).

Not only were alveolar macrophages more sensitive to LPS, but they were also more responsive to low concentrations of glycine. Glycine inhibited transient increases in $[Ca^{2+}]_i$ due to LPS in isolated alveolar macrophages in this study (Figs. 2 and 3) in a dose-dependent manner but with an IC_{50} value >10-fold less than values reported for Kupffer cells (16). This difference may also be explained by differences in receptor density or by a downregulation event due to levels of exposure of each cell type to glycine. Perhaps glycine-sensitive chloride channels are expressed more abundantly in alveolar macrophages as opposed to Kupffer cells. Alternatively, preliminary long-term studies suggest that the effect of glycine on LPS-induced increases in $[Ca^{2+}]_i$ in Kupffer cells is lost after chronic exposure to dietary glycine (Wheeler, unpublished data). This suggests that chronic elevated glycine concentrations possibly lead to glycine receptor downregulation or desensitization in Kupffer cells, causing the effect of glycine to be lost or reduced. This possibility is likely, since Kupffer cells are continuously exposed to blood concentrations of glycine of 100 μM or higher, and alveolar macrophages exist in the alveolar space where the glycine concentration is presumably lower than in blood. In fact, glycine concentrations in alveolar fluid (50–60 μM) were significantly lower than in the blood (90–160 μM). Thus differences in local glycine concentrations may explain differences in sensitivity of various macrophage populations to glycine.

Alveolar macrophages contain a glycine-gated chloride channel. The data above suggest that alveolar macrophages possess a glycine-gated chloride channel. The hypothesis is strongly supported by the fact that the effects of glycine are both sensitive to strychnine and are dependent on the presence of extracellular chloride (Figs. 4 and 6). First, glycine blunted the activation of the alveolar macrophage with an IC_{50} slightly greater than 10 μM, which is near the binding affinity of glycine for the glycine-gated chloride channel in neuronal tissue (37). Strychnine at low concentrations is a known antagonist of the glycine-gated chloride channel in nervous tissue and has been useful in pharmacological and biochemical studies of the channel in various tissues and expression systems. In the spinal cord, strychnine binds and inhibits the activity of the channel with nanomolar affinity; however, strychnine mimics glycine by activating chloride movement through the channel at higher concentrations (23, 24). The effects of strychnine in alveolar macrophages (Fig. 4) are consistent with this hypothesis, providing pharmacological evidence for the existence of the glycine receptor with similar characteristics to the channel found in both neuronal and renal tissue. All of the pharmacological data such as strychnine sensitivity, extracellular chloride dependence, and radiolabeled chloride influx are consistent with the hypothesis that glycine activates a glycine-gated chloride channel similar to the channel in neuronal tissue. Moreover, molecular evidence for a glycine-gated chloride channel in alveolar macrophages using reverse transcription-polymerase chain reaction has recently been obtained (data not shown).

Glycine modulates the production of free radicals and cytokines in alveolar macrophages. The increase in $[Ca^{2+}]_i$ due to LPS serves as a second messenger in many signaling pathways and is important in the production of free radicals and some inflammatory cytokines that are released by macrophages (14, 28). These studies demonstrate that glycine significantly reduces the LPS-induced production of $O_2^-\cdot$ and TNF-α in alveolar macrophages, most likely by blunting the increase in $[Ca^{2+}]_i$ necessary for their production. $O_2^-\cdot$ from macrophages is largely generated from molecular O_2 through NADPH oxidase, an enzyme complex activated by phosphorylation by calcium-dependent protein kinases (4, 38). By blunting the increase in $[Ca^{2+}]_i$ with glycine, the production of $O_2^-\cdot$ is reduced most

likely by inhibiting calcium-dependent signaling required to activate NADPH oxidase (Fig. 7).

TNF-α production is stimulated by LPS and is dependent on an increase in $[Ca^{2+}]_i$, which is involved in signaling and protein synthesis. LPS is known to activate macrophages through CD14, β_2-integrins, and scavenger receptors (9). In long-term in vitro assays, such as the measurement of TNF-α, both CD14 and scavenger receptor pathways most likely become activated and initiate cytokine production. Even though there is a significant effect of glycine on LPS-induced TNF-α production, glycine is not able to completely blunt LPS-induced TNF-α production like $[Ca^{2+}]_i$ and $O_2^-\cdot$ production. The reason for these differences is not understood yet but may be due to the involvement of scavenger receptors or other receptors and pathways that may signal via receptors independent of CD14. In fact, it is reported that TNF-α production in response to long-term exposure to LPS can increase independent of the transient increase in $[Ca^{2+}]_i$ (19). Together, these data suggest that it is likely that several receptors and pathways are involved in LPS-induced TNF-α production. In this case, glycine would be expected to have minimal effects. On the other hand, the transient LPS-induced increase in $[Ca^{2+}]_i$ and $O_2^-\cdot$ production is most likely mediated entirely through CD14, since it occurs within minutes. In this case, glycine is most effective.

The inhibition of free radical and TNF-α production by glycine is an important phenomenon, since others have shown that mediators produced by alveolar macrophages play a large role in the pathological changes in sepsis, ARDS, and possibly asthma (5, 34). Moreover, these data may provide an additional explanation for the protective effect of dietary glycine on endotoxin-induced mortality and lung injury (15), since alveolar macrophages play a large role in lung pathogenesis in that model.

Moreover, alveolar macrophages play a significant role in the inflammatory process after exposure to inhaled particles such as cotton dust, a rich source of endotoxin, or other organic dusts (33). TNF-α production from alveolar macrophages mediates a cascade of events including histamine release, neutrophil and lymphocyte inflammation, and potential chronic lung injury. Because glycine inactivated alveolar macrophages and blunted the production of TNF-α, glycine may potentially be useful in the treatment or prevention of lung inflammation due to inhaled particles.

In conclusion, it was shown that glycine-gated chloride channels are present in alveolar macrophages and can prevent activation by allowing the protective influx of extracellular chloride, which opposes increases in $[Ca^{2+}]_i$. Because increases in $[Ca^{2+}]_i$ are required for free radical production as well as production of many cytokines in alveolar macrophages, glycine may be useful in preventing tissue injury due to inflammatory agents in response to LPS or other antigens. Because many diseases such as sepsis, ARDS, bronchial allergies, and acute inflammatory responses are mediated, in part, by alveolar macrophage activation, glycine may be especially useful in their treatment if shown to be effective in clinical trials.

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REFERENCES

1. **Bautista, A. P., and J. J. Spitzer.** Cross-tolerance between acute alcohol-intoxication and endotoxemia. *Alcohol. Clin. Exp. Res.* 20: 1395–1400, 1996.
2. **Beutler, B., and A. Cerami.** The biology of cachectin/TNF-α primary mediator of the host response. *Annu. Rev. Immunol.* 7: 625–655, 1989.
3. **Burgess, A. W., and D. Metcalf.** The nature and action of granulocyte-macrophage colony stimulating factors. *Blood* 56: 947–958, 1980.
4. **Castagna, M., Y. Takai, K. Sano, U. Kikawa, and Y. Nishizuka.** Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257: 7847–7851, 1982.
5. **Chabot, F., J. A. Mitchell, J. M. C. Gutteridge, and T. Evans.** Reactive oxygen species in acute lung injury. *Eur. Respir. J.* 11: 745–757, 1998.
6. **Drysdale, B.-E., R. A. Yapundich, M. L. Shin, and H. S. Shin.** Lipopolysaccharide-mediated macrophage activation: the role of calcium in the generation of tumoricidal activity. *J. Immunol.* 138: 951–956, 1987.
7. **D'Souza, N. B., G. J. Bagby, S. Nelson, C. H. Lang, and J. J. Spitzer.** Acute alcohol infusion suppresses endotoxin-induced serum tumor necrosis factor. *Alcohol. Clin. Exp. Res.* 13: 295–298, 1989.
8. **Dubin, W., T. R. Martin, P. Swoveland, D. J. Leturcq, A. M. Moriarty, P. S. Tobias, E. R. Bleeker, S. E. Goldblum, and J. D. Hasday.** Asthma and endotoxin: lipopolysaccharide-binding protein and soluble CD14 in bronchoalveolar compartment. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L736–L744, 1996.
9. **Fenton, M. J., and D. T. Golenbock.** LPS-binding proteins and receptors. *J. Leukoc. Biol.* 64: 25–32, 1998.
10. **Finkel, T. H., M. J. Pabst, H. Suzuki, L. A. Guthrie, J. R. Forehand, W. A. Phillips, and R. B. Johnston, Jr.** Priming of neutrophils and macrophages for enhanced release of superoxide anion by the calcium ionophore ionomycin. Implications for regulation of the respiratory burst. *J. Biol. Chem.* 262: 12589–12596, 1987.
11. **Goya, T., M. Abe, H. Shimura, and M. Toriso.** Characteristics of alveolar macrophages in experimental septic lung. *J. Leukoc. Biol.* 52: 236–243, 1992.
12. **Haak-Frendscho, M., N. Arai, K. Arai, M. L. Beaza, A. Finn, and A. P. Kaplan.** Human recombinant granulocyte-macrophage colony stimulating factor and interleukin-3 cause basophil histamine release. *J. Clin. Invest.* 82: 17–20, 1988.
13. **Hijioka, T., R. L. Rosenberg, J. J. Lemasters, and R. G. Thurman.** Kupffer cells contain voltage-dependent calcium channels. *Mol. Pharmacol.* 41: 435–440, 1991.
14. **Hotchkiss, R. S., W. M. Bowling, I. E. Karl, D. F. Osborne, and M. W. Flye.** Calcium antagonists inhibit oxidative burst and nitrite formation in lipopolysaccharide-stimulated rat peritoneal macrophages. *Shock* 8: 170–178, 1997.
15. **Ikejima, K., Y. Iimuro, D. T. Forman, and R. G. Thurman.** A diet containing glycine improves survival in endotoxin shock in the rat. *Am. J. Physiol.* 271 (*Gastrointest. Liver Physiol.* 34): G97–G103, 1996.
16. **Ikejima, K., W. Qu, R. F. Stachlewitz, and R. G. Thurman.** Kupffer cells contain a glycine-gated chloride channel. *Am. J. Physiol.* 272 (*Gastrointest. Liver Physiol.* 35): G1581–G1586, 1997.

17. **Kaplan, A. P., M. Haak-Frendscho, A. Fauci, C. Dinarello, and E. Halbert.** A histamine-releasing factor from activated human mononuclear cells. *J. Immunol.* 135: 2027–2032, 1985.
18. **Lane, S. J., A. R. Sousa, and T. H. Lee.** The role of the macrophage in asthma. *Allergy* 49: 201–209, 1994.
19. **Lichtman, S. N., J. Wang, and J. J. Lemasters.** Lipopolysaccharide-stimulated TNF-α release from cultured rat Kupffer cells: sequence of intracellular signaling pathways. *J. Leukoc. Biol.* 64: 368–372, 1998.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** Protein measurement with the Folin phenol regent. *J. Biol. Chem.* 193: 265–275, 1951.
21. **Martin, T. R., J. C. Mathison, P. S. Tobias, D. J. Leturcq, A. M. Moriarty, R. J. Maunder, and R. J. Ulevitch.** Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implication for cytokine production in normal and injured lungs. *J. Clin. Invest.* 90: 2209–2219, 1992.
22. **McCord, J. M., and I. Fridovich.** The utility of superoxide dismutase in studying free radical reactions. II. The mechanism of the mediation of cytochrome *c* reduction by a variety of electron carriers. *J. Biol. Chem.* 245: 1374–1377, 1970.
23. **Miller, G. W., E. A. Lock, and R. G. Schnellmann.** Strychnine and glycine protect renal proximal tubules from various nephrotoxicants and act in the late phase of necrotic cell injury. *Toxicol. Appl. Pharmacol.* 125: 192–197, 1994.
24. **Miller, G. W., and R. G. Schnellmann.** Cytoprotection by inhibition of chloride channels: the mechanism of action of glycine and strychnine. *Life Sci.* 53: 1211–1215, 1993.
25. **Morrow, A. L., and S. M. Paul.** Benzodiazepin enhancement of γ-aminobutyric acid mediated Cl^- ion flux in rat brain synaptoneurosomes. *J. Neurochem.* 50: 302–306, 1988.
26. **Norwood, S. H., and J. K. Ceretti.** The adult respiratory syndrome. *Surg. Gynecol. Obstet.* 161: 497–508, 1985.
27. **Ogle, C. K., W. Jun-Zhen, M. Xialing, K. Szczur, W. Alexander, and J. D. Ogle.** Heterogeneity of Kupffer cell and splenic, alveolar and peritoneal macrophages for the production of TNF, IL-1, and IL-6. *Inflammation* 18: 511–523, 1994.
28. **Park, Y. C., C. D. Jun, H. S. Kang, H. D. Kim, H. M. Kim, and H. T. Chung.** Role of intracellular calcium as a priming signal for the induction of nitric oxide synthesis in murine peritoneal macrophages. *Immunology* 87: 296–302, 1996.
29. **Parsons, P. E., F. A. Moore, E. E. Moore, D. N. Ikle, P. M. Henson, and G. S. Worthen.** Studies on the role of tumor necrosis factor in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 146: 694–700, 1992.
30. **Parsons, P. E., G. S. Worthen, E. E. Moore, R. M. Tate, and P. M. Henson.** The association of circulating endotoxin with the development of the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 140: 294–301, 1989.
31. **Prpic, V., J. E. Weiel, S. D. Somers, J. DiGuiseppi, S. L. Gonias, S. V. Pizzo, T. A. Hamilton, B. Herman, and D. O. Adams.** Effect of bacterial lipopolysaccharide on the hydrolysis of phosphatidylinositol-4,5-biphosphate in murine peritoneal macrophages. *J. Immunol.* 139: 526–533, 1987.
32. **Rajendra, S., J. W. Lynch, and P. R. Schofield.** The glycine receptor. *Pharmacol. Ther.* 73: 121–146, 1997.
33. **Ryan, L. K., and M. H. Karol.** Release of tumor necrosis factor in guinea pigs upon acute inhalation of cotton dust. *Am. J. Respir. Cell. Mol. Biol.* 5: 93–98, 1991.
34. **Smith, P. D., A. F. Suffredini, J. B. Allen, L. M. Wahl, J. E. Parrillo, and S. M. Wahl.** Endotoxin administration to human primes alveolar macrophages for increased production of inflammatory mediators. *J. Clin. Invest.* 14: 141–148, 1994.
35. **Weinberg, J. M., J. A. Davis, M. Abarzua, and T. Rajan.** Cytoprotective effects of glycine and glutathione against hypoxic injury to renal tubules. *J. Clin. Invest.* 80: 1446–1454, 1987.
36. **Wheeler, M. D., R. F. Stachlewitz, and R. G. Thurman.** Glycine blunts alveolar macrophage activation by a mechanism involving a glycine-gated chloride channel (Abstract). *Toxicologist* 37: 346, 1998.
37. **Young, A. B., and S. H. Snyder.** Strychnine binding associated with glycine receptors of the central nervous system. *Proc. Natl. Acad. Sci. USA* 70: 2832–2836, 1973.
38. **Zhou, H., R. F. Duncan, T. W. Robison, L. Gao, and H. J. Forman.** Ca^{2+}-dependent $p47^{phox}$ translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. *Am. J. Physiol.* 273 (*Lung Cell. Mol. Physiol.* 17): L1042–L1047, 1997.