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Review

Glycine: a new anti-inflammatory immunonutrient

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Abstract. The mechanism of the immunosuppressive effects of glycine and its pathophysiological applications are discussed in this review. Glycine has been well characterized in spinal cord as an inhibitory neurotransmitter which activates a glycine-gated chloride channel (GlyR) expressed in postsynaptic membranes. Activation of the channel allows the influx of chloride, preventing depolarization of the plasma membrane and the potentiation of excitatory signals along the axon. Glycine has recently been shown to have similar inhibitory effects on several white blood cells, including hepatic and alveolar macrophages, neutrophils, and lymphocytes. Pharmacological analysis using a GlyR antagonist strychnine, chloride-free buffer, and radiolabeled chloride has provided convincing evidence to support the hypothesis that many white blood cells contain a glycine-gated chloride channel with properties similar to the spinal cord GlyR. Molecular analysis using reverse transcription-polymerase chain reaction and Western blotting has identified the mRNA and protein for the β subunit of the GlyR in total RNA and purified membrane protein from rat Kupffer cells. Dietary glycine is protective in rat models against endotoxemia, liver ischemia-reperfusion, and liver transplantation, most likely by inactivating the Kupffer cell via this newly identified glycine-gated chloride channel. Glycine also prevents the growth of B16 melanomas cell in vivo. Moreover, dietary glycine is protective in the kidney against cyclosporin A toxicity and ischemia-reperfusion injury. Glycine may be useful clinically for the treatment of sepsis, adult respiratory distress syndrome, arthritis, and other diseases with an inflammatory component.

Key words. Glycine; immunoregulation; anti-inflammatory; glycine receptor.

General introduction

Glycine has long been known to be an inhibitory neurotransmitter in the spinal cord [1]. Glycine-mediated inhibitory neurotransmission is essential for startle responses, voluntary motor control and sensory signal processing in the spinal cord [2–4]. Glycine exerts its inhibitory actions by binding its receptor (GlyR) which is largely localized in postsynaptic neuronal membranes [5]. Inhibitory postsynaptic signals oppose the depolarizing action of excitatory/stimulatory neurotransmission by increasing chloride permeability across the postsynaptic neuronal membrane. The identity of glycine as an inhibitory neurotransmitter was originally proposed by Aprison et al. [6] and Davidoff et al. [4], who described in detail the distribution of glycine throughout the central nervous system. Autoradiographic studies with radiolabeled glycine demonstrated that glycine is localized in spinal cord synaptic regions [7]. Functional studies later demonstrated that glycine hyperpolarizes postsynaptic motor neurons by increasing

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chloride conductance [3, 8, 9]; thus, the receptor for glycine is often referred to as a glycine-gated chloride channel. Inhibitory neurotransmission by glycine was shown to be selectively blocked by strychnine, a plant alkaloid, which enabled further characterization of glycine action in the nervous system [10, 11]. With the use of the high-affinity inhibitor strychnine, the GlyR was purified from membrane fractions of the adult rat spinal cord [12, 13]. The subunit composition and binding sites of the receptor and the amino acid sequence of many of the subunits have been characterized (reviewed by Rajendra et al., ref. [1]).

Recently, work from our group has demonstrated that a wide variety of white blood cells involved in inflammation (i.e., Kupffer cells, alveolar macrophages, and neutrophils) also contain glycine-gated chloride channels [14–16]. By hyperpolarizing the plasma membrane of leukocytes, glycine makes them less sensitive to inflammatory stimuli such as endotoxin and possibly a wide variety of growth factors. Since glycine is one of the amino acids in serum that declines in shock, the immunoregulatory role of glycine may be very important. Moreover, elevation of blood levels of glycine with simple dietary administration has shown remarkable improvement in shock [17], alcoholic liver injury [18], some forms of cancer [19], and nephrotoxicity due to certain drugs [20]. The purpose of this article, therefore, is to review recent evidence on the beneficial effects of glycine.

Role of calcium in signaling in Kupffer cells

Calcium is central in cellular regulation, and its basic physiological and biochemical properties have been studied for decades [21]. Only recently, with advances in molecular characterization of calcium channels and fluorescent indicators sensitive enough to track intracellular movement of calcium, have advances become exponential. A survey of the entire area is beyond the scope of this review; however, interested readers are referred to Berridge [22] and Putney [23]. Briefly, the plasma membrane of mammalian cells contains two classes of calcium channels, which are either responsive to agonists and antagonists (receptor-operated channels) or regulated by membrane potential (voltage-operated channels). The former involves production of inositol 1,4,5-triphosphate (IP_3) via G-protein-linked mechanisms triggered by binding of the agonist (e.g., epinephrine) to the receptor which releases Ca^{2+} from intracellular stores (reviewed by Putney et al., ref. [24]). The opening of the latter channel is dependent on the membrane potential of the plasma membrane which is a function of distribution of ions in the basal state. Collectively, these channels are involved in muscle contraction, release of neurotransmitters, regulation of cell proliferation, apoptosis, and cell differentiation.

It is known that Kupffer cells, the resident hepatic macrophages, are activated by calcium, but conclusive evidence that they contain voltage-dependent calcium channels has only recently been demonstrated in this laboratory [25]. By measuring the cytosolic free calcium concentration ($[Ca^{2+}]_i$) of cultured Kupffer cells, replacement of extracellular Na^+ by K^+ caused an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner, most likely due to membrane depolarization. Further, increases in intracellular calcium via influx of calcium through voltage-dependent calcium channels can be induced by stimulating Kupffer cells with endotoxin (LPS). The exact mechanism whereby LPS causes an increase in $[Ca^{2+}]$ is not completely understood. One possible mechanism is that LPS generates a signal in Kupffer cells by activating its receptor CD14. CD14 is associated with a transmembrane protein toll-like receptor (tlr 2/4), which may serve as a tyrosine kinase similar to the intracellular region of the interleukin (IL)-1 receptor [26]. The hypothesis is that initiation of this signaling pathway by LPS leads to the activation of phospholipase C and the generation of IP_3 causing the release of calcium from intracellular stores. Simultaneously, depolarization of the plasma membrane causes Ca^{2+} influx, but the mechanisms for this are poorly understood. The change in the membrane potential activates voltage-operated calcium channels causing the influx of extracellular calcium [27, 28]. The dramatic increase in intracellular free calcium then serves as a second-messenger signal for cellular signaling events, cell mobilization, and transcription and translation of key cytokines (see fig. 1).

L-type Ca^{2+} channels in Kupffer cells

Hijioki et al. [25] showed that K^+-induced Ca^{2+} influx in Kupffer cells was sensitive to the L-type Ca^{2+} channel blocker, nisoldipine. Moreover, it was shown that Ca^{2+} influx induced by the Ca^{2+} channel agonist BAY K 8644 was also inhibited by nitrendipine. Thus it was hypothesized that Kupffer cells contained L-type Ca^{2+} channels. In support of this idea, Takei et al. [29] showed that the L-type Ca^{2+} channel blocker nisoldipine improves graft survival after cold storage and transplantation of rat liver, actions that were confirmed in isolated perfused rabbit liver [30]. We also found that nisoldipine decreased tumor necrosis factor-α (TNF-α) release from liver after transplantation in this model [31]. Patients with alcoholic hepatitis or cirrhosis frequently exhibit endotoxemia, leading to the production of TNF-α by Kupffer cells. Therefore, modulation of Kupffer cell function by blocking Ca^{2+} channels represents a new approach to treatment of hepatic pathology-

ical conditions such as alcoholic liver injury, and improvement in procedures such as liver transplantation.

Characteristics of glycine-gated chloride channels

The GlyR was first purified by Pfeiffer and Betz [12] using high-affinity strychnine binding from rat spinal cord. The channel is comprised of three distinct protein subunits: a 48-kDa α subunit, a 58-kDa β subunit, and a 93-kDa cytoplasmic anchoring protein, gephyrin [12, 13]. Three different isoforms of the α subunit have been identified and cloned from rat: the original purified 48-kDa α subunit (α1), a 49-kDa α2 subunit [32], and a 50-kDa α3 subunit [33, 34]. Moreover, homologues of the α1, α2, and the β subunits of the GlyR have been identified and cloned from human and mouse spinal cord [35–40]. Recently, a fourth α subunit has been identified (denoted α4) by Matzenbach et al. [41]. For rat, mouse, and human, the α subunits share striking sequence identity with each other and with subunits of the nicotinic acetylcholine receptor (nAchR) and the GABA type A receptor ($GABA_AR$), as well as several other ligand-gated chloride channels [1, 33]. The GlyR is comprised of five subunits, formed from either α subunits or a combination of α and β subunits, arranged in a pentameric complex which spans the cell membrane. The cytoplasmic region of the β subunit forms a complex with the anchoring protein gephyrin. The functional properties of the GlyR are related to the subunit composition of the complete pentamer. Generally, subunit composition is dictated by either developmental stage or region of expression.

In functional analysis of cultured or isolated in vitro spinal cord preparations, the EC_{50} values for glycine activation range from 20 to 150 μM [42, 43]. These values are usually consistent with those obtained with recombinant GlyR expressed in mammalian cells [44, 45]. However, most recombinant GlyRs have significantly less sensitivity to glycine (EC_{50} 0.3–1 mM) [34,

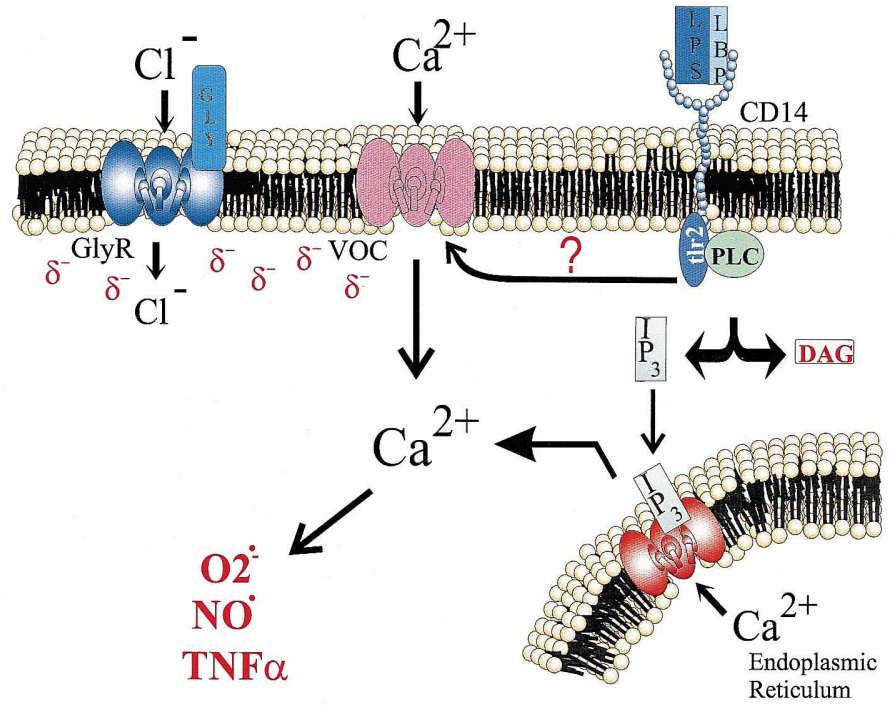

Figure 1. Working hypothesis. Glycine activates a ligand-gated chloride channel in the plasma membrane of Kupffer cells which causes an influx of chloride ions leading to the hyperpolarization of the membrane. Upon an external stimulus such as endotoxin, voltage-dependent influx of extracellular free calcium occurs through voltage-operated channels. This increase in intracellular calcium is blunted due to the hyperpolarized state of the plasma membrane by chloride. Intracellular signaling and cytokine production which is dependent upon the increase in intracellular calcium are blunted, preventing the cascade of inflammatory cytokines following activation of Kupffer cells and other white blood cells which contain the glycine receptor. (DAG, diacylglycerol; Glyr, glycine receptor; IP_3, inositol 1,4,5-triphosphate; LPS, lipopolysaccharide; PLC, phospholipase c; tlr-2, toll-like receptor-2; VOC, voltage-operated calcium channel; TNFα, tumor necrosis factor-α; LBP, lipopolysaccharide binding protein).

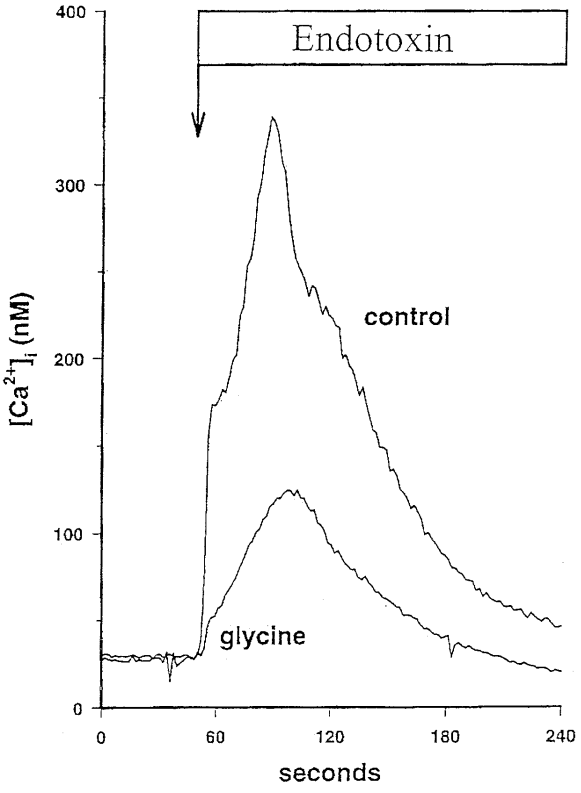

Figure 2. Effect of glycine on lipopolysaccharide (LPS)-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated Kupffer cells. $[Ca^{2+}]_i$ in a cultured Kupffer cell was measured fluorometrically using the fluorescent Ca^{2+} indicator fura-2 [14]. LPS was added to stimulate the increase in $[Ca^{2+}]_i$, while 1000 μM glycine was added 3 min before LPS.

36, 46, 47]. This is important since blood glycine levels can be elevated over the EC_{50} values simply by dietary treatment. Interestingly, the GlyR can also be activated by β-alanine and taurine; however, they are less potent than glycine [48].

Pharmacological evidence for glycine-gated chloride channels in Kupffer cells

Strychnine, chloride-free buffer, and membrane potential

Based on studies with strychnine, chloride-free buffer, and measurement of radioactive chloride flux, it has been shown that Kupffer cells contain a glycine-gated chloride channel [14, 18]. First, the effect of glycine on $[Ca^{2+}]_i$ in cultured Kupffer cells stimulated with LPS was investigated to assess whether they contain a glycine-gated chloride channel. LPS increased $[Ca^{2+}]_i$ rapidly with peak values reaching over 300 nM (fig. 2). Glycine (1 mM) prevented this increase nearly completely. Moreover, low concentrations of strychnine (1 μM), an antagonist to the GlyR in the central nervous system, reversed the inhibitory effect of glycine completely. The effect of glycine was prevented when cells were incubated in chloride-free buffer. To test the hypothesis that glycine-gated chloride channels hyperpolarize the plasma membrane of Kupffer cells, changes in plasma membrane potential were estimated in individual Kupffer cells using the voltage-sensitive dye, bis-oxonol (fig. 3). High potassium (25 mM), which depolarizes the plasma membrane, increased fluorescence intensity, indicating that changes in fluorescence indeed reflect changes in membrane potential. Glycine (1 mM) gradually decreased fluorescence within 2 min and blunted increases in fluorescence caused by potassium. Thus, it was concluded that glycine hyperpolarizes the plasma membrane, making depolarization more difficult. Further, LPS increased fluorescence intensity transiently, an effect also blunted by glycine. This indicates that LPS causes depolarization of the Kupffer cell plasma membrane and that the effect can be prevented by glycine. To evaluate the effect of glycine on cytokine

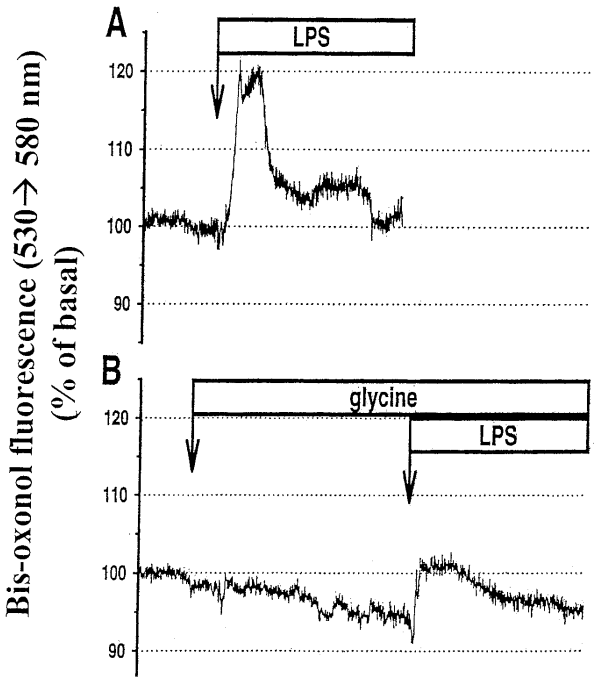

Figure 3. The effect of glycine on Kupffer cell membrane potential. The relative membrane potential of isolated Kupffer cells was measured fluorometrically using the fluorescent voltage-sensitive dye bis-oxonol. (*A*) LPS was added to induce membrane depolarization of Kupffer cells. (*B*) Glycine (1 mM) was added 3 min prior to LPS.

Table 1. Evidence that a wide variety of white cells contain glycine-gated chloride channels.

Cell type	Agonist-induced increase in $[Ca^{2+}]_i$ blocked by glycine	IC_{50} value (mM)	Reversal by strychnine	Dependence of extracellular chloride	Glycine-stimulated $^{36}Cl^-$ influx
Kupffer cell	+	~0.3–0.6	+	+	+
Alveolar macrophage	+	~0.1	+	+	+
Neutrophil	+	0.3–0.5	+	+	+
Jurkat	+		+	+	
T lymphocyte	+				
Blood monocyte	+				

Blanks indicate that the particular parameter has not been studied.

production by Kupffer cells, LPS-induced TNF-α production was measured. As expected, isolated Kupffer cells produced large amounts of TNF-α in the presence of LPS (1 μg/ml). However, glycine (1 mM) reduced LPS-induced TNF-α production by about 70%. This effect of glycine on TNF-α production was also reversed by low-dose strychnine (1 μM). In comparison to the GlyR in the spinal cord where the IC_{50} for glycine is 30–100 μM [48], the IC_{50} for glycine on the Kupffer cell is slightly higher (~0.3 mM). Conversely, GlyRs expressed in in vitro expression systems displayed much lower sensitivity to glycine (IC_{50}, 0.3–1 mM) [34]. It was concluded therefore from this pharmacological evidence that Kupffer cells contain a glycine-gated chloride channel similar to one described previously in the spinal cord. Prevention of increases in $[Ca^{2+}]_i$ due to LPS by activation of chloride influx reduces synthesis and release of toxic cytokines by Kupffer cells.

Glycine stimulates chloride flux in Kupffer cells

In synaptosomes, influx of radiolabeled chloride is used to measure the flux of chloride through chloride channels [49]. Therefore, we reasoned that if Kupffer cells contain a glycine-gated chloride channel, glycine would stimulate movement of radiolabeled chloride [18]. Indeed, glycine stimulated chloride movement with EC_{50} values between 0.1 and 0.5 mM. This provides hard physical evidence that Kupffer cells contain a glycine-gated chloride channel.

Taurine

Since taurine, a ubiquitous sulfur-containing β-amino acid, acts like glycine in neurons by causing hyperpolarization, it was hypothesized that taurine would act via a mechanism similar to that of glycine and blunt the LPS-induced increase in $[Ca^{2+}]_i$ in Kupffer cells. To test this hypothesis, Kupffer cells were isolated from rats and cultured for 24 h. LPS-induced changes in $[Ca^{2+}]_i$ were monitored fluorometrically in single cells, while levels of TNF-α released by Kupffer cells after exposure to LPS were measured by ELISA. Taurine significantly blunted the LPS-induced increase in $[Ca^{2+}]_i$ in a dose-dependent manner (IC_{50}, 0.11 mM), the IC_{50} being similar to that for its action on the spinal cord GlyR (IC_{50}, 0.05–0.1 mM) [50]. This effect was reversed by strychnine (1 μM) and was prevented when chloride was removed from the extracellular medium. Moreover, like glycine, taurine increased $^{36}Cl^-$ uptake by Kupffer cells in a dose-dependent manner. In contrast, other sulfur-containing amino acids (i.e., cysteine and methionine) were without effect. These results support the hypothesis that taurine, like glycine, activates a chloride channel in Kupffer cells. In addition, LPS-induced TNF-α production was reduced by more than 40% by taurine, an effect which was also reversed by strychnine. Thus, taurine blocks the increase in $[Ca^{2+}]_i$ due to LPS and significantly reduces TNF-α production by mechanisms involving chloride influx into the Kupffer cell.

Glycine-gated chloride channels appear to be ubiquitous in leukocytes

One important question is whether the glycine-gated chloride channel is unique to Kupffer cells or whether it exists in other leukocytes. In the six types of leukocytes we have studied to date (see table 1), glycine blunted agonist-induced increases in $[Ca^{2+}]_i$, a phenomenon dependent on extracellular chloride and reversed by strychnine. Further, glycine and taurine stimulated radiolabeled chloride flux in all leukocytes studied so far, providing good physical evidence to support the idea that glycine-gated chloride channels are widespread in leukocyte populations. Recently, Spittler et al. [51] showed that glycine inhibited TNF-α and IL-1 production and enhanced expression of IL-10 from isolated blood monocytes, further confirming the immunosuppressive effects of glycine.

Molecular evidence for glycine-gated chloride channels in Kupffer cells

Since the GlyR in the central nervous system is a pentameric assembly of four subtypes of ligand-binding α subunits and a single subtype of a structural β subunit, mRNA from isolated Kupffer cells was reverse transcribed and amplified using primers specific for an internal region of the glycine receptor β subunit. RT-PCR amplification from Kupffer cell mRNA resulted in a 550-base-pair fragment as predicted from the cloned sequence from the spinal cord glycine receptor (fig. 4). PCR amplification of the GlyR β subunit from spinal cord cDNA also resulted in a 550-base-pair fragment. The low quantity of the mRNA in Kupffer cells compared to spinal cord may reflect the relative expression of the receptor in the different tissues. It is likely that Kupffer cells express much less GlyR than spinal cord. Alternatively, the minute quantity may be due to inefficient amplification of the cDNA in Kupffer cells, resulting from slightly different nucleotide sequences. Since the primers for RT-PCR were designed based on the spinal cord GlyR sequence, differences in the nucleotide sequence in the Kupffer cell GlyR mRNA would lower PCR amplification efficiency.

The GlyR in the brain has been successfully detected using the monoclonal antibody, anti-GlyR4a, which recognizes regions on both α and β subunits of the receptor [35]. Western blot analysis of Kupffer cell membranes using the GlyR4a antibody yielded evidence for both α and β subunits; however, the proteins were slightly larger than the subunits detected in rat spinal cord membranes [52].

Molecular evidence has been presented for both subunits of the GlyR in the central nervous system [36], and Western blotting has identified the β subunit in the kidney tubule [53]. RT-PCR and Western blotting for the glycine-gated chloride channel in the Kupffer cell provide molecular evidence for the existence of the receptor in Kupffer cells. However, the molecular weights of the proteins detected in the Kupffer cell with the anti-GlyR4a monoclonal antibody differ from those of the α and β subunits of the spinal cord GlyR. However, the molecular weight of the amplified RT-PCR product from the Kupffer cell was as predicted (fig. 4). Therefore, it was hypothesized that the two proteins identified in the Kupffer cell are highly similar to the α and β subunits of cloned GlyR in specific regions, such as ligand-binding sites or transmembrane-spanning regions, but are somehow different in overall structure and/or sequence, thus yielding larger proteins than expected. Differences in molecular weights may be due to posttranslational modifications of the subunits. There are three putative N-glycosylation sites on both the α and β subunits. Whether or not there are modifications which contribute to the discrepancy in molecular weights is not known. However, these data provide the first molecular evidence that Kupffer cells indeed possess a glycine-gated chloride channel similar to that expressed in neuronal tissue.

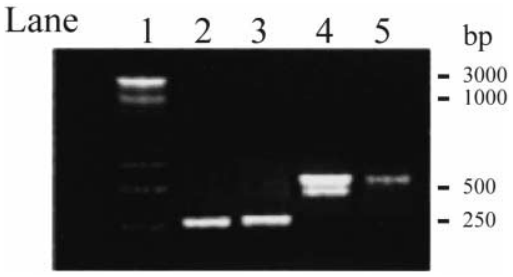

Figure 4. Reverse transcription-polymerase chain reaction (RT-PCR) for the glycine receptor β subunit in Kupffer cells. RNA isolated from Kupffer cells was subjected to RT-PCR using PCR primers specific for the glycine receptor β subunit, with β actin as control. Spinal cord RNA was also used as a positive control. Lane 1, 100-base-pair DNA ladder; 2, spinal cord β actin; 3, Kupffer cell β actin; 4, spinal cord glycine receptor β subunit; 5, Kupffer cell glycine receptor β subunit.

Examples of beneficial effects of glycine

For many, it is difficult to fathom that beneficial effects can be obtained in several disease states with the simplest amino acid, glycine. However, evidence continues to mount in favor of this idea. It is now clear that dietary glycine, which increases the blood concentration of glycine to greater than 1 mM from basal concentrations ranging from 0.1–0.2 mM, protects against shock caused either by blood loss or endotoxin. It reduces alcohol levels in the stomach and improves recovery from alcoholic hepatitis [54]. It also reduces fibrosis caused by experimental drugs. It diminishes liver injury caused by hepatotoxic drugs and blocks programmed cell death [55]. Moreover, it reduces cancer caused by chemicals [56]. In the kidney, it reduces the nephrotoxicity caused by the drug cyclosporin A and prevents hypoxia and free radical formation [57]. However, we predict that it will be useful in other diseases because it (i) diminishes TNF-α production and (ii) decreases cell signaling via a unique mechanism.

Unique concept of glycine action

The question then arises as to how glycine has such beneficial effects. The answer is that it most likely has an inhibitory effect on cell signaling mechanisms in cells that contain a glycine-gated chloride channel. As men-

tioned above, receptor- and voltage-gated calcium channels are central in elevation of calcium for intracellular signaling in many immune cell types such as the Kupffer cell. Furthermore, it is known that increases in $[Ca^{2+}]_i$ trigger opening of a chloride channel in the plasma membrane leading to hyperpolarization, making voltage-dependent calcium channels more difficult to open. We hypothesize that glycine opens a chloride channel in the plasma membrane of Kupffer cells and other white blood cells, rendering calcium influx triggered by a variety of agonists, drugs, and growth factors more difficult or impossible. In vitro data from isolated Kupffer cells clearly support this hypothesis and explain the near universal action of glycine [14]. Thus, many other disease states involving activated immune cells, in particular macrophages, neutrophils, and lymphocytes, should be affected by elevated levels of glycine, according to our hypothesis.

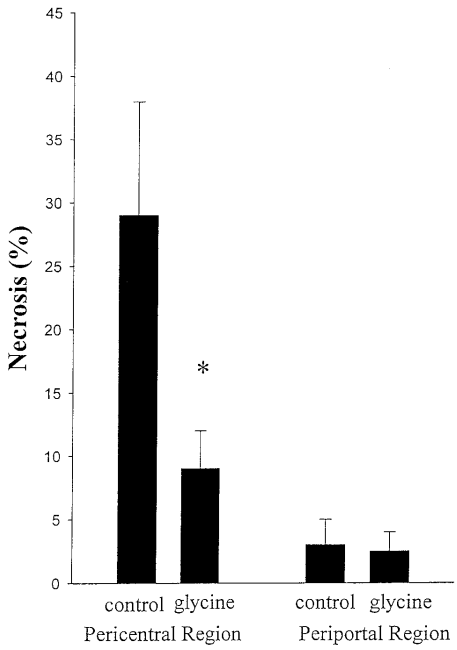

Figure 5. Effect of glycine on rates of cell death in periportal and pericentral regions of the liver lobule following reperfusion. Livers were perfused for 45 min at low-flow rates (1 ml/min). Glycine (2 mM final concentration) was infused 10 min prior to reperfusion at normal flow rates (4 ml/min). Trypan blue was infused at the end of 40 min of reperfusion. Nuclei of parenchymal cells in a zone radiating five cells from either pericentral or periportal regions were identified as trypan blue positive or negative. The percentage of staining was calculated from the number of stained nuclei divided by the total number of cells in a region. Values are means $\pm$ SE ($n = 4-5$, ANOVA, $P = 0.001$). *$P < 0.05$ compared with control group.

Reperfusion injury and surgical manipulation

Because glycine prevents cell death induced by anoxia in proximal tubules of the kidney, we studied its effect on hypoxia-reoxygenation in the liver. We used a low-flow, reflow liver perfusion model [58]. With this protocol, livers were perfused at low flow rates of ~ 1 ml/g per minute for 75 min, which caused cells in pericentral regions of the liver lobule to become anoxic due to insufficient delivery of oxygen. When normal flow rates (~ 4 ml/g per minute) were restored for 40 min, oxygen-dependent reperfusion injury occurred. Upon reflow, lactate dehydrogenase (LDH), a cytosolic enzyme, and malondialdehyde (MDA), an end product of lipid peroxidation, were released into the effluent perfusate. LDH increased from basal levels of ~ 1 to 35 IU/g per hour in livers from control rats. Glycine (0.06–2.00 mM) minimized enzyme release in a dose-dependent manner (half-maximal decrease = 133 μM), with maximal values reaching only 5 IU/g per hour when glycine was increased to 2 mM. Reflow for 40 min after 75 min of low-flow hypoxia caused death in $\sim$ 30% of previously anoxic parenchymal cells in pericentral regions; however, infusion of glycine decreased cell death to less than 10% (fig. 5). Trypan blue distribution time, an indicator of hepatic microcirculation, was reduced significantly by glycine at 5 and 40 min after reflow. Time for oxygen to reach steady state upon reflow was reduced by glycine in a dose-dependent manner, and the rates of entry and exit of a dye confined to vascular space (fluorescein dextran) were increased two- to threefold by glycine, respectively. Taken together, these data indicate that reperfusion injury that occurs in previously hypoxic pericentral regions of the liver upon reintroduction of oxygen is minimized by glycine, most likely by action on a glycine-sensitive anion channel which improves microcirculation during the reperfusion period, possibly by decreasing the production of vasoactive cytokines and eicosanoids from Kupffer cells.

Next, we wanted to see if this information on reperfusion injury and glycine could be applied to a clinically relevant problem. For this purpose, we chose primary nonfunction associated with liver transplantation [59, 60]. The etiology of primary graft nonfunction and dysfunction is still unknown but most likely involves Kupffer-cell-dependent reperfusion injury; however, recent evidence indicates that the donor operation may also be important [60]. Moreover, treatment with gadolinium chloride, a Kupffer cell toxicant [61], depletes the liver of Kupffer cells and reduces liver dysfunction following transplantation, clearly supporting the hypothesis that Kupffer cells participate in primary nonfunction [60]. The role of manipulation of the liver which cannot be prevented completely during standard harvesting techniques was assessed (fig. 6). Donor rat

livers were harvested either with or without gentle manipulation. Subsequently, orthotopic liver transplantation was performed after 1 h of cold storage in University of Wisconsin cold storage solution. In some rats, Kupffer cells were treated with dietary glycine before harvest. In the nonmanipulated group, survival was 100%; however, gentle manipulation decreased survival to ~30% after transplantation. Further, manipulation elevated transaminases five-fold 8 h after transplantation. Dietary glycine prevented the effects of organ manipulation on all parameters studied. These data indicate for the first time that brief, gentle manipulation of the liver has a marked detrimental effect on survival by mechanisms involving Kupffer cells.

Endotoxin shock

The effects of a glycine-containing diet (5%) on mortality and liver injury due to intravenous injection of LPS were studied in rats in vivo (fig. 7A) [17]. Fifty percent of the rats fed the control diet died within 24 h after an intravenous injection of LPS (10 mg/kg), whereas feeding rats glycine totally prevented mortality and markedly reduced the LPS-induced elevation of serum transaminases, hepatic necrosis, and lung injury. The elevation in serum TNF-α due to LPS was also blunted and delayed significantly by glycine feeding (fig. 7B). In a two-hit model (hepatic ischemia and injection of sublethal LPS), all rats fed control diet died, whereas 83% of glycine-fed animals survived with a significant reduc-

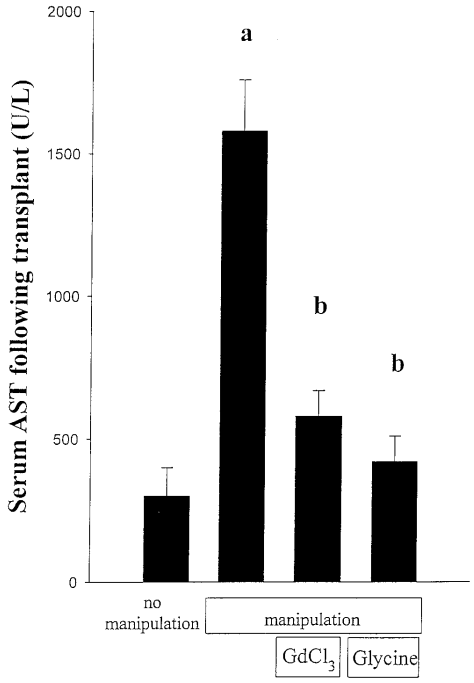

Figure 6. Effects of gentle organ manipulation on serum aspartate transaminase (AST) levels following transplantation. Animals were untreated or treated with gadolinium chloride (10 mg/kg) or a 5% glycine diet for 3 days prior to surgery. Blood was collected 8 h after transplantation for serum AST measurement. Values are means ± SE (n = 4–8, two-way ANOVA, P = 0.05). [a]P < 0.05 compared with no manipulation; [b]P < 0.05 compared with manipulation without pretreatment.

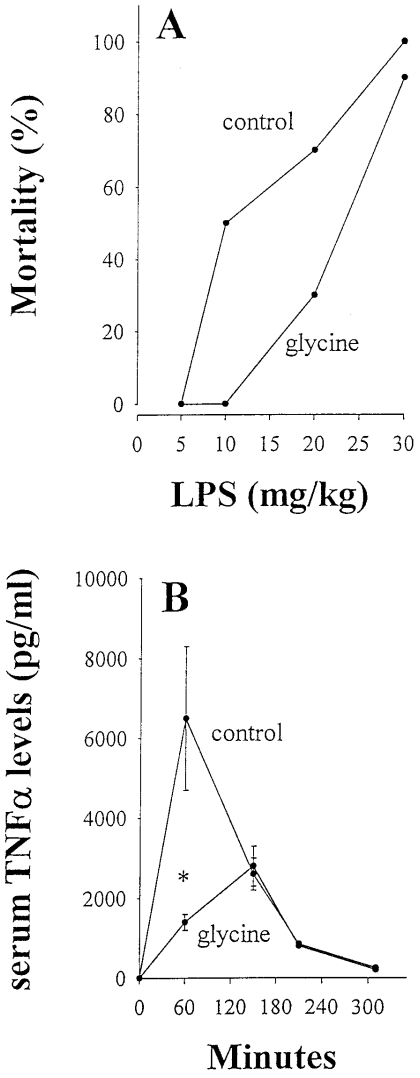

Figure 7. Effect of dietary glycine on survival and TNF-α production due to endotoxin shock. Data are means ± SE (n = 6, *P < 0.05 with Mann-Whitney's rank-sum test). (*A*) Rats were fed a 5% glycine or control diet for 3 days prior to the injection of endotoxin (5–30 mg/kg). Survival rates were monitored for 24 h. (*B*) Rats were fed a 5% glycine or control diet for 3 days prior to endotoxin injection (10 mg/kg). Serum TNF-α levels were monitored for up to 310 min.

tion in transaminases and improved liver and lung histology. LPS elevated $[Ca^{2+}]_i$ in cultured Kupffer cells, an effect blocked almost completely by glycine [14]. Glycine most likely reduces injury and mortality by preventing the LPS-induced elevation of $[Ca^{2+}]_i$ in Kupffer cells, thereby minimizing toxic eicosanoid and cytokine (e.g., LTB_4 and TNF-α) production.

Alcohol

Tsukamoto-French model. When Kupffer cells were inactivated with gadolinium chloride ($GdCl_3$) or endotoxin was minimized by antibiotics, early alcohol-induced liver injury was blocked in experimental animals. Since glycine also inactivates Kupffer cells, we explored its actions in alcohol-induced liver injury [54]. Male Wistar rats were exposed to ethanol (10–12 g/kg per day) continuously for up to 4 weeks via an intragastric feeding protocol. The effect of glycine on the first-pass metabolism of ethanol was also examined in vivo, and the effect on alcohol metabolism was estimated specifically in perfused liver. Glycine decreased ethanol concentrations precipitously in urine, breath, peripheral blood, portal blood, feces, and stomach contents. Moreover, serum aspartate aminotransferase levels were elevated to 183 U/l after 4 weeks of ethanol treatment. In contrast, values were significantly lower in rats given glycine along with ethanol. Hepatic steatosis and necrosis were also reduced significantly by glycine. Glycine dramatically increased the first-pass elimination of ethanol in vivo but had no effect on alcohol metabolism in the perfused liver. Thus, it is concluded that glycine minimizes alcohol-induced liver injury in vivo, preventing ethanol from reaching the liver by accelerating first-pass metabolism by the stomach [54, 62].

Recovery from alcoholic hepatitis. Since the effect of glycine on ethanol metabolism in the stomach complicated the evaluation of the effect of glycine in alcohol-induced liver disease, we studied its effect on the recovery phase [18]. When patients with alcoholic liver disease enter hospital, alcohol is removed. Accordingly, we induced alcoholic hepatitis with the Tsukamoto-French protocol for 6 weeks. Then, either control or glycine-containing diets were given and recovery from liver injury was assessed. In as little as 1 week, liver histology and serum transaminases were improved over 30% by a glycine-containing diet compared to a control diet. Correspondingly, TNF-α mRNA was reduced more in liver tissue by glycine than in controls, most likely by increasing the Cl^- flux into Kupffer cells, thereby diminishing cytokine and eicosanoid production. These experiments are very important, since they suggest that simple dietary glycine, which should be well tolerated by patients, could speed recovery from alcoholic hepatitis.

Cancer

Peroxisome proliferator WY-14,643-induced hepatocyte proliferation. Peroxisome proliferators are a group of nongenotoxic carcinogens which include a number of hypolipidemic drugs, solvents, and industrial plasticizers. Although the mechanism by which they cause cancer remains unknown, one likely possibility is that they act as tumor promoters by increasing cell proliferation [63]. Kupffer cells represent a rich source of mitogenic cytokines (e.g., TNF-α) and have been shown to be stimulated by peroxisome proliferators [64]. Since glycine prevents activation of Kupffer cells, these experiments were designed to test the hypothesis that a diet containing glycine could prevent the mitogenic effect of the peroxisome proliferator WY-14,643 (fig. 8) [56]. The effects of glycine on WY-14,643-induced increases in cell proliferation after a single dose or after feeding WY-14,643 in the diet (0.1%) for three weeks were assessed. As expected, 24 h after a single dose of WY-14,643, rates of cell proliferation were increased about

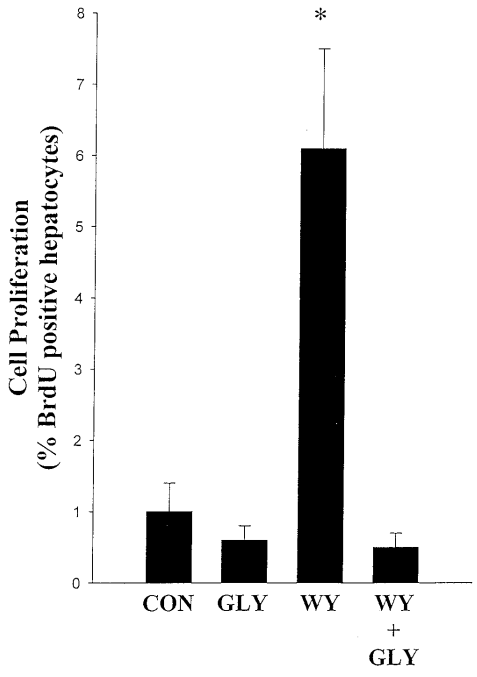

Figure 8. Cell proliferation after 3 weeks of WY-14,643 (WY) and glycine (GLY) in the diet. Cell proliferation was assessed by BrdU incorporation. Values are means ± SE [n = 5, two-way ANOVA, *P < 0.05, compared with control (CON) and WY + GLY groups].

fivefold. Glycine largely prevented the increase in cell turnover. Acyl CoA oxidase, a marker enzyme for peroxisomes, increased significantly, indicating that peroxisomes were induced about twofold in livers of WY-14,643-treated rats after 24 h. Unlike cell proliferation, however, acyl CoA oxidase was not affected by glycine, consistent with the hypothesis that cell and peroxisome proliferation results from different signaling pathways. After 3 weeks, dietary glycine reduced basal rates of cell proliferation by about 50% and completely prevented the sustained fivefold increase in cell proliferation caused by feeding dietary WY-14,643. Thus, weeks of dietary exposure to WY-14,643 caused a sixfold increase in acyl CoA oxidase activity which was also unaffected by glycine, demonstrating that a diet containing glycine prevents the increase in hepatocyte proliferation caused by a potent peroxisome proliferator without affecting induction of peroxisomes. These data support the hypothesis that dietary glycine could be effective in preventing cancer caused by nongenotoxic carcinogens such as WY-14,643.

These data are consistent with the hypothesis that production of TNF-α by Kupffer cells plays a central role in the development of peroxisome-proliferator-induced liver cancer and raises the possibility that Kupffer cells may also be important in the development of cancer caused by other nongenotoxic carcinogens. The complete prevention of WY-14,643-induced cell proliferation and the 50% reduction in basal levels of hepatocyte replication with a diet containing glycine predicts that glycine may be an effective dietary tool for the prevention and possibly even the treatment of cancer.

Tumors from B-16 melanoma cells. Since dietary glycine inhibited hepatocyte proliferation in response to WY-14,643 [56] and cell replication is associated with hepatic cancer caused by WY-14,643 [65], glycine may have general anticancer properties. Therefore, the hypothesis that glycine would inhibit the growth of tumors arising from implanted B16 melanoma cells was tested [19]. Mice were fed a diet containing 5% glycine and 15% casein or a control diet containing 20% casein. After monitoring tumor volume daily for 14 days, the tumor was removed, weighed, and sectioned for histological analysis (fig. 9). Tumors from glycine-fed mice weighed 65% less than those from control animals after 14 days; however, neither tumor size nor mitotic index differed 2 days after implantation when tumor growth was independent of vascularization. Further, tumors from mice fed glycine had fewer arteries after 14 days, suggesting an inhibitory role of glycine on angiogenesis and tumor vascularization. Indeed, glycine (0.01–10 mM) inhibited the growth of endothelial cells in vitro, supporting the hypothesis that glycine inhibits tumor growth *in vivo* through mechanisms involving endothelial cell proliferation.

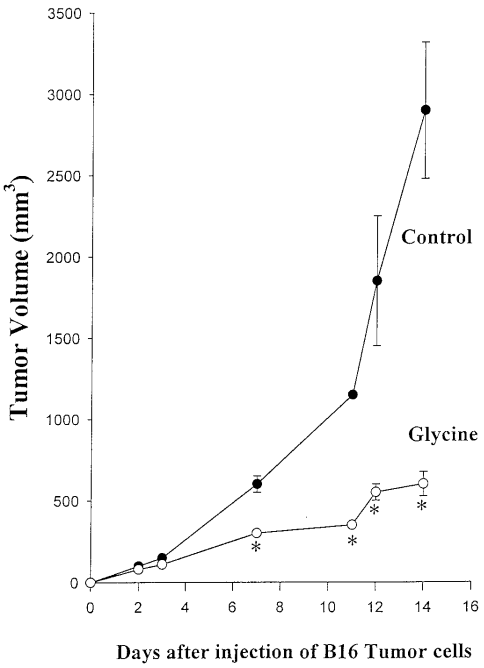

Figure 9. Effect of dietary glycine on tumor volume. Tumor diameter was measured using digital calipers and the volume was calculated. Data shown are means $\pm$ SE (n = 5, repeated-measures ANOVA on ranks, *$P < 0.05$).

Nephrotoxicity

Prevention of cyclosporin-A-induced nephrotoxicity with dietary glycine. The nonessential amino acid glycine has been used previously to prevent hypoxic and ischemic injury to kidney tissue in vitro [53, 66, 67]. Since there is some evidence that the immunosuppressant cyclosporin A causes nephrotoxicity through a hypoxia-reoxygenation mechanism that could involve infiltration and activation of macrophages and neutrophils, we hypothesized that dietary glycine could prevent this injury (fig. 10). Accordingly, rats were fed a diet containing glycine (5%) or a control diet for 3 days prior to cyclosporin A treatment. To produce nephrotoxicity, cyclosporin A (25 mg/kg daily by gavage) was administered for 28 days while animals were maintained on glycine or control diets. Serum creatinine and urea, glomerular filtration rates, and kidney histology were evaluated. As expected, cyclosporin A caused kidney damage in rats fed the control diet, reflected in significantly elevated serum urea and creatinine. In addition, cyclosporin A treatment decreased glomerular filtration rates by nearly 70%, caused proximal tubular dilation and necrosis as well as increased macrophage and neutrophil infiltration into the kidney. Dietary glycine pre-

vented or minimized kidney damage due to cyclosporin A in all parameters studied. Furthermore, feeding glycine for up to 1 month had no detrimental effect on kidney function. Dietary glycine is a safe and effective treatment to reduce the nephrotoxicity of cyclosporin A.

One major advantage of glycine over drug therapy is that it most likely acts at several points in the pathology due to cyclosporin A by preventing vasoconstriction, proximal tubular hypoxia, as well as activation of macrophages and mesangial cells. It is hypothesized that glycine inactivates macrophages by blunting the increase in $[Ca^{2+}]_i$, thereby minimizing the release of vasoactive and inflammatory eicosanoids and cytokines. A second advantage is that glycine is a natural, nontoxic amino acid circulating in the 100–200 μM range under normal conditions. Thus, dietary supplementation of cyclosporin A patients with glycine should be of immense benefit in preventing the major side effect of nephrotoxicity.

Role of hypoxia and free radicals in cyclosporin A toxicity. It is likely that cyclosporin A causes vasoconstric-

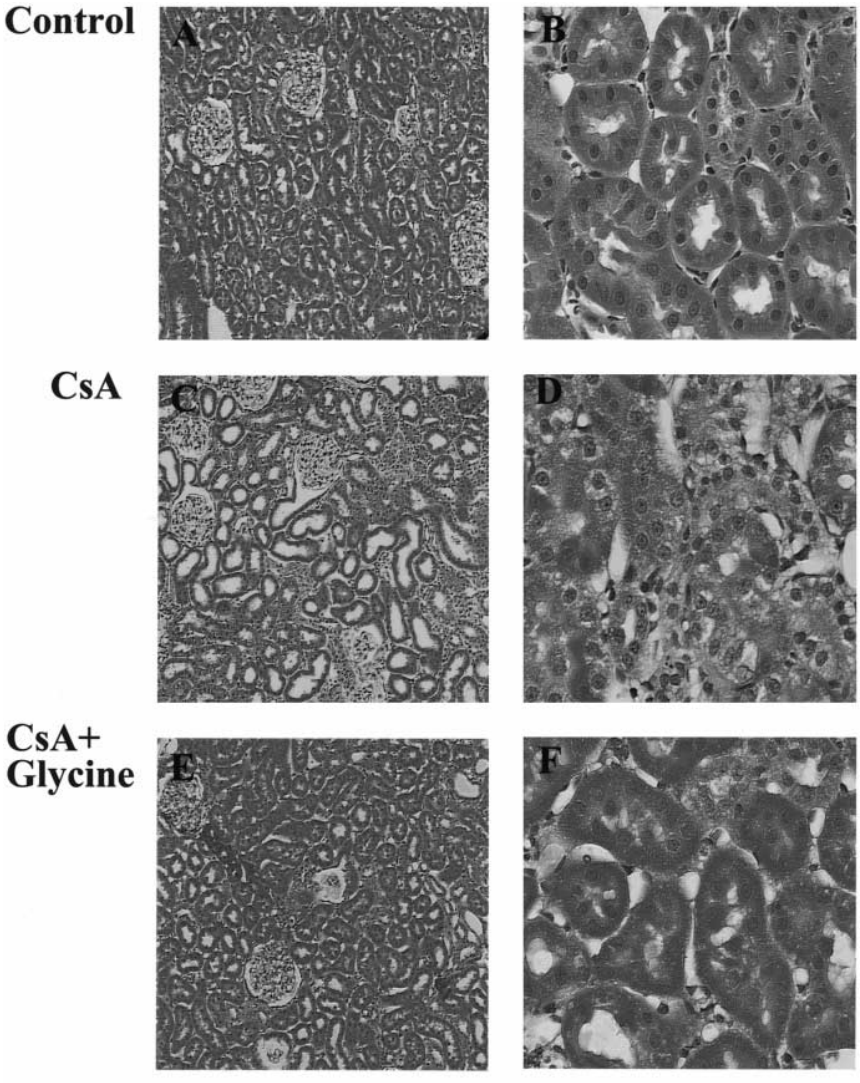

Figure 10. Prevention of kidney pathology due to cyclosporin A (CSA)with glycine. Photomicrographs of perfusion-fixed kidneys from rats after 4 weeks of treatment at low (*A*,*C*,*E*) (× 100) and high (*B*,*D*,*F*) (× 400) magnification. (*A*,*B*) Sections from control animals. (*C*,*D*) Animals treated for 4 weeks with cyclosporin (25 mg/kg). (*E*,*F*) Sections from animals treated for 4 weeks with cyclosporin (25 mg/kg) fed a glycine-containing (5%) diet.

tion which leads to hypoxia-reperfusion injury; therefore, experiments were designed to attempt to obtain physical evidence for hypoxia and free radical production in kidney following cyclosporin A treatment. Rats were treated daily with cyclosporin A (25 mg/kg, p.o.) for 5 days, and pimonidazole, a 2-nitroimidazole hypoxia marker, was injected 2 h after the last dose of cyclosporin A. α-(4-Pyridl 1-oxide)-*N*-*tert*-butylnitrone (POBN) was injected 3 h after cyclosporin A to trap free radicals. Cyclosporin A nearly doubled serum creatinine and decreased glomerular filtration rates by 65% as expected. Pimonidazole adduct binding in the kidney was increased nearly threefold by cyclosporin A, providing physical evidence for tissue hypoxia. Moreover, cyclosporin A increased POBN/radical adducts nearly sixfold in the urine but did not alter levels in the serum. Glycine, an amino acid which prevents cyclosporin A toxicity, minimized cyclosporin A-induced hypoxia, blocked free radical production, and blunted decreases in glomerular filtration rate. These results demonstrate clearly for the first time that cyclosporin A causes hypoxia in renal cells and increases production of a new free radical species exclusively in the kidney. Therefore, it is concluded that cyclosporin A causes renal injury by mechanisms involving hypoxia-reoxygenation. Moreover, these effects can be prevented effectively by dietary glycine.

Clinical considerations

Based on these exciting findings, it is reasonable to propose that glycine would be useful in the treatment of many inflammatory-type diseases in humans. Obvious disease targets include sepsis and endotoxemia, experienced in many patients following abdominal surgery or trauma. It is also reasonable to think that glycine may be useful in many respiratory diseases such as adult respiratory distress syndrome and asthma. Moreover, the use of glycine in the prevention and/or treatment of certain types of cancer looks promising. As the mechanisms of tumorigenesis and angiogenesis become clearer, the role of glycine as an anticancer agent may become more exciting. Certainly, the prospect of pre- and posttransplant treatment with glycine in combination with standard immunosuppressive agents is an exciting possibility. Since common immunosuppressive agents exhibit many toxic effects, cotreatment with glycine may ameliorate these effects, allowing doses of several toxic drugs to be lowered. All the ramifications of dietary glycine treatment have certainly not been addressed, but the practical implications of the diet have been surprising. A long-term 5% glycine diet (> 4 weeks) still proves beneficial in some models. Additionally, lowering the glycine composition to 1.25% in the diet is sufficient to protect tissues in several models. These considerations should stimulate further investigation into clinical applications of dietary glycine. Because glycine can be elevated simply by dietary measures, the feasibility of therapeutic and preventive approaches for many diseases with this new immunonutrient is quite promising.

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