L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent

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Purpose of review

In recent years, evidence has mounted in favor of the antiinflammatory, immunomodulatory and cytoprotective effects of the simplest amino acid L-glycine. This article will focus on the recent findings about the responsible mechanisms of protection and review the beneficial effects of glycine in different disease states.

Recent findings

Glycine protects against shock caused by hemorrhage, endotoxin and sepsis, prevents ischemia/reperfusion and cold storage/reperfusion injury to a variety of tissues and organs including liver, kidney, heart, intestine and skeletal muscle, and diminishes liver and renal injury caused by hepatic and renal toxicants and drugs. Glycine also protects against peptidoglycan polysaccharide-induced arthritis and inhibits gastric secretion and protects the gastric mucosa against chemically and stress-induced ulcers. Glycine appears to exert several protective effects, including antiinflammatory, immunomodulatory and direct cytoprotective actions. Glycine acts on inflammatory cells such as macrophages to suppress activation of transcription factors and the formation of free radicals and inflammatory cytokines. In the plasma membrane, glycine appears to activate a chloride channel that stabilizes or hyperpolarizes the plasma membrane potential. As a consequence, agonist-induced opening of L-type voltage-dependent calcium channels and the resulting increases in intracellular calcium ions are suppressed, which may account for the immunomodulatory and antiinflammatory effects of glycine. Lastly, glycine blocks the opening of relatively non-specific pores in the plasma membrane that occurs as the penultimate event leading to necrotic cell death.

Summary

Multiple protective effects make glycine a promising treatment strategy for inflammatory diseases.

Keywords

glycine, inflammation, cytoprotection, immune response

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Abbreviations

FMLP	formyl-methionine-leucine-phenylalanine
GlyR	glycine receptor
IP_3	inositol 1,4,5 triphosphate
NOS	nitric oxide synthase
PCR	polymerase chain reaction
PG-PS	peptidoglycan polysaccharide
PGE_2	prostaglandin E_2
ROS	reactive oxygen species
TNF	tumor necrosis factor

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Introduction

L-Glycine is a simple, non-essential amino acid that consists of a single carbon molecule attached to an amino and a carboxyl group. Glycine is an inhibitory neurotransmitter in the central nervous system. In non-nervous tissue, glycine is often considered biologically neutral and is used as an isonitrogenous control in studies of supplementation with other amino acids. However, much evidence has accumulated that glycine is an effective antiinflammatory, immunomodulatory and cytoprotective agent. Glycine protects in a variety of disease states in experimental models such as ischemia–reperfusion injury, shock, transplantation, alcoholic hepatitis, hepatic fibrosis, arthritis, tumor and drug toxicity. Preliminary data from clinical trials also show promising effects on human liver grafts after transplantation. The mechanisms by which glycine protects remain incompletely understood. Proposed mechanisms include suppression of calcium signaling, inhibition of inflammatory cell activation, decreased formation of free radicals and other toxic mediators, and blockage of plasma membrane permeabilization preceding oncotic necrosis. Thus, glycine may exert its protective effect by multiple mechanisms with additive or synergistic effects. This article will discuss the responsible mechanisms of protection and review the beneficial effects of glycine in different disease states.

Mechanisms of protection by glycine

The protective effects of glycine are probably due to its direct effect on target cells or mediated by inhibition of inflammatory cell activation. The underlying mechanisms are not totally clear. Several mechanisms have been proposed.

Activation of the glycine-gated chloride channel and modulation of calcium channels

Activation of the glycine-gated chloride channel is one widely postulated mechanism for the effects of glycine.

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Glycine is an inhibitory neurotransmitter in the central nervous system. Glycine exerts its inhibitory actions by binding the glycine receptor (GlyR) which is localized largely in the postsynaptic neuronal membranes of the spinal cord. Activation of GlyR leads to increases in chloride conductance [1]. Therefore, GlyR is also called the glycine-gated chloride channel. An influx of chloride hyperpolarizes postsynaptic membranes, thus counteracting the depolarizing action of excitatory neurotransmitters. Strychnine, a plant alkaloid, selectively blocks the effect of glycine on this receptor at low concentration but can act as a partial agonist at high concentrations [2]. GlyR is composed of three types of subunits: α, a ligand binding subunit; β, a structural subunit and gephyrin, a cytoplasmic anchoring protein [3]. Five subunits, either all α-subunits or a combination of α- and β-subunits, form a pentameric complex which spans the cell membrane. The cytoplasmic region of the β-subunit binds to gephyrin [3]. The EC_{50} value of GlyR activation by glycine in spinal cord preparations ranges from 20 to 150 μM. Glutathione ($Ki = 10$ μM) and cysteinyl-glycine, but not oxidized glutathione, can also activate GlyR, an effect that is blocked by strychnine [4]. GlyR also exists in a wide variety of cells other than neurons. Cells involved in inflammatory and immune responses, such as macrophages, monocytes, neutrophils, Jurkat cells, immortalized human lymphoblast cells, and T lymphocytes, possess GlyR [5,6••,7–9]. Hepatocytes, endothelial cells and renal proximal tubular cells also show the presence of GlyR [10•,11••,12,13••]. Glycine blocks the increases in intracellular calcium ions in these cells caused by many structurally different stimuli. These stimuli include endotoxin, peptidoglycan polysaccharide (PG-PS), bacterial-derived peptide formyl-methionine-leucine-phenylalanine (FMLP), bile acids, D-galactosamine, peroxisome proliferators, cyclosporin A, prostaglandin E_2 (PGE_2), phenylephrine, vascular endothelial growth factor, concanavalin A, and anti-CD_3 antibody [5,6••,7–9,14,15]. The increased intracellular calcium caused by these stimuli is dependent on extracellular chloride and is blocked by glycine. Inhibition of calcium increases by glycine is associated with influx of radiolabeled chloride. Strychnine, the selective inhibitor of the neuronal glycine-gated chloride channel, reverses the effects of glycine, supporting the conclusion that these cells contain a glycine-gated chloride channel (Fig. 1).

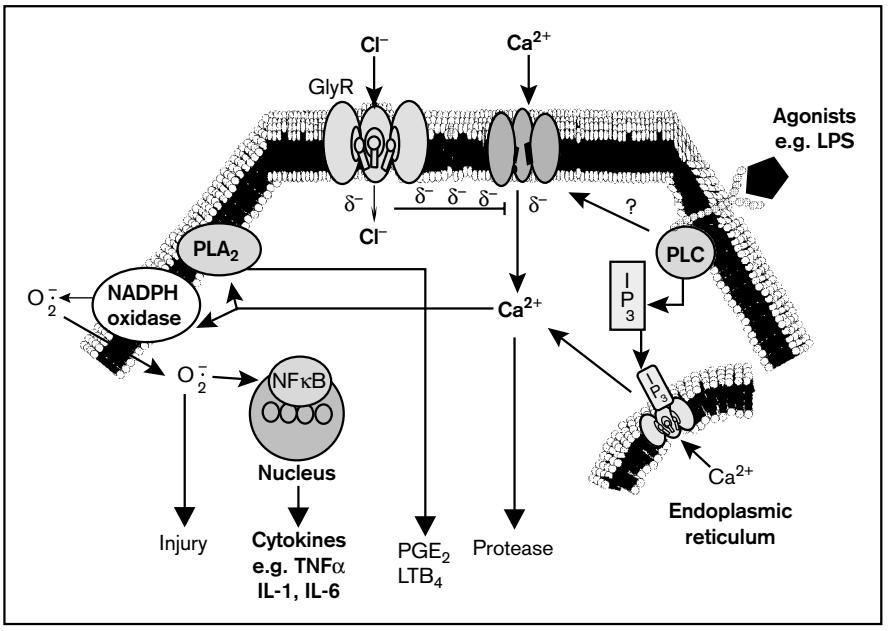
Calcium plays an important role in cellular regulation. The exact mechanism by which glycine blocks increased intracellular calcium levels by calcium-mobilizing agonists is not completely understood. These agonists come from diverse different classes, and they bind to different receptors. Thus, it is unlikely that glycine acts by blocking each of these receptors. In general, agonists binding to receptors activate phospholipase C and generate inositol 1,4,5 triphosphate (IP_3), which induces release of calcium from intracellular stores. Simultaneously, depolarization of the plasma membrane opens voltage-dependent calcium channels to allow an influx of extracellular calcium (Fig. 1). Increased intracellular calcium signals a variety of intracellular events. These events include production of cytokines and inflammatory mediators, activation of enzymes such as protease and phospholipase A, cell proliferation and cell death. Macrophage activation leads to production of reactive oxygen and nitrogen species. Activation of GlyR suppresses these calcium-dependent events by inducing an influx of chloride and consequent hyperpolarization of cell membranes, and the inhibition of voltage-dependent opening of calcium channels (Fig. 1).

Glycine receptors in non-neuronal cells

The presence of GlyR in many inflammatory cells likely mediates the inhibition by glycine of inflammatory and immune responses. Glycine suppression of proinflammatory responses also occurs in cells other than white blood cells. Vascular endothelial growth factor increases intracellular calcium in cultured vascular endothelial cells, and this effect is blocked by glycine [11••]. Glycine also inhibits the proliferation and migration of endothelial cells and smooth muscle cells [11••,16], suggesting that glycine may be beneficial in inhibiting graft rejection, cardiovascular disease and angiogenesis. Recent work suggested that liver parenchymal cells also contain GlyR. In isolated hepatocytes, glycine blocks the increase in intracellular calcium due to PGE_2 and phenylephrine, an α_1-type adrenergic receptor agonist [10•]. Low-dose strychnine partially reverses the inhibition by glycine. When extracellular chloride is omitted, glycine is much less effective in preventing increases in intracellular calcium due to PGE_2. These data suggest that hepatoprotection by glycine is, in part, due to its direct effect on hepatocytes via regulating intracellular calcium [10•].

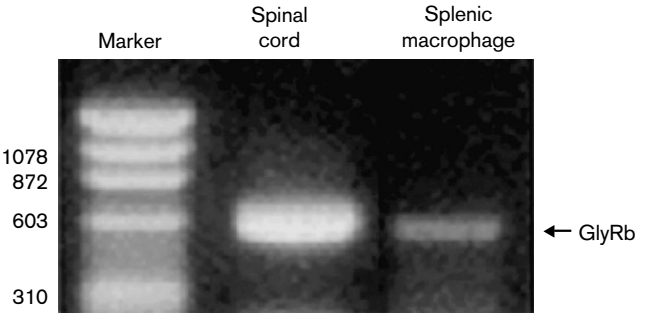
In support of pharmacological evidence for the existence of GlyR in non-neuronal cells, recent studies provide molecular evidence for GlyR in non-neuronal cells. Reverse transcriptase polymerase chain reaction (PCR) using primers specific for conserved regions in the β-subunit identifies GlyR in isolated splenic macrophages and vascular endothelial cells (Fig. 2). The PCR product from splenic macrophages has 98% homology with a complementary DNA segment of the β-subunit of GlyR [6••]. Proteins corresponding to the β-subunit of the strychnine-sensitive GlyR and the associated protein gephyrin were also identified in rabbit kidney cortical membrane fractions and renal proximal tubules [12]. In purified membranes from Kupffer cells, peritoneal neutrophils and splenic and alveolar macrophages membranes, approximately 48 kDa α-subunits of GlyR

Figure 1. Proposed mechanism for inhibition of agonist-induced increases in intracellular calcium by glycine



In general, agonists such as endotoxin (lipopolysaccharide; LPS) and peptidoglycan polysaccharide (PG-PS), bind to corresponding receptors, thus leading to activation of phospholipase C (PLC) and production of inositol 1,4,5 triphosphate (IP_3) via G-protein-linked mechanisms. IP_3 induces the release of calcium from intracellular stores. Simultaneously, plasma membrane depolarization activates voltage-operated calcium channels and causes influx of extracellular calcium. Increases in intracellular calcium serve as a signal for a variety of intracellular events. For example, activation of phospholipase A_2 (PLA_2) leads to formation of arachidonic acid and production of vasoactive and chemotacttic mediators such as prostaglandins and leukotrienes. Activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase leads to reactive oxygen species production that not only causes cell injury directly but also activates transcription factors, such as nuclear factor (NF) κB, and production of proinflammatory and mitogenic cytokines, such as tumor necrosis factor (TNF)α and interleukins (ILs) 1 and 6. Glycine activates a glycine-gated chloride channel (GlyR) in the plasma membrane to cause influx of chloride ions, which hyperpolarizes the cell membrane. It is hypothesized that hyperpolarization decreases the open probability of calcium channels, thereby blocking movement of calcium across the plasma membrane and subsequent events in the inflammatory process.

Figure 2. Glycine receptor messenger RNA in splenic macrophages



Splenic macrophages were isolated from non-treated rats, and RNA was extracted with phenol:chloroform and ethanol precipitation. The glycine receptor (GlyR) was identified by reverse transcriptase polymerase chain reaction (PCR) using primers designed from the cloned sequence of highly conserved regions in the β-subunit of the GlyR from rat spinal cord. The PCR product was confirmed by sequencing. The data are representative of three independent experiments. Messenger RNA of Gly$R\beta$ was detected in the RNA from the spinal cord and splenic macrophages. Adapted with permission [6••].

are present, as detected by Western blot using the anti-GlyR-α antibody against receptor purified from the spinal cord [17••].

Although GlyR is present in Kupffer cells, peritoneal neutrophils, and splenic and alveolar macrophages, α-subunits of the receptor in these cells are not the same. Reverse transcriptase PCR for each subtype of the α-subunit shows that fragments for α1- and α4-subunits are present in Kupffer cells whereas fragments for α2- and α4-subunits are present in neutrophils and splenic and alveolar macrophages [17••]. Additionally, the GlyR β-subunit is present in Kupffer cells, neutrophils and splenic and alveolar macrophages, as detected by reverse transcriptase PCR [17••]. The sequence of the cloned receptor fragment (approximately 550 base pairs) is over 95% homologous with the nucleotide sequence of the GlyR β-subunit from adult rat spinal cord. Ribonuclease protection assays of RNA from Kupffer cells, splenic macrophages, alveolar macrophages, and neutrophils show the predicted GlyR template fragment [17••].

These findings suggest that GlyR exists in these cells but that GlyR α-subunit heterogeneity exists within the monocyte/macrophage cell lineage population, possibly creating a functional diversity between these cells. Importantly, recent studies demonstrated that long-term glycine exposure blunts activation of alveolar macrophages and neutrophils by lipopolysaccharide but not of Kupffer cells [7], suggesting that differences in the GlyR subunits may lead to differential regulation of desensitization. In contrast to pharmacological evidence for GlyR in hepatocytes [10•], molecular evidence for GlyR in hepatocytes has not been found [17••].

Effects of glycine on free radicals, inflammatory mediator production, and degradative enzyme activation

Glycine decreases oxidative stress in many disease states [18,19,20••,21,22••]. Reactive oxygen species (ROS) can damage biologically important macromolecules such as lipids, DNA and protein, directly leading to cell injury [23,24]. In addition, ROS activate phospholipase A_2, which increases production of lipid-derived vasoactive and chemotactic mediators such as eicosanoids and platelet activating factor [25]. Oxidative stress also activates transcription factors such as nuclear factor-κB and activator protein-1, leading to synthesis of proinflammatory cytokines and cell adhesion molecules [23]. Reactive oxygen production mediates endotoxin-induced nuclear factor-κB activation and tumor necrosis factor (TNF)α production [26•,27•]. ROS arise from a number of sources. Certain chemicals and drugs can give rise to ROS through their metabolism. Reoxygenation after ischemia promotes ROS formation by xanthine oxidase-mediated metabolism of purine derivatives accumulated after ATP degradation and by ubisemiquinone-linked reactions at complexes I and III of the mitochondrial respiratory sequence [24]. In addition, activated macrophages and neutrophils produce oxygen radicals via reduced nicotinamide adenine dinucleotide phosphate oxidase [24].

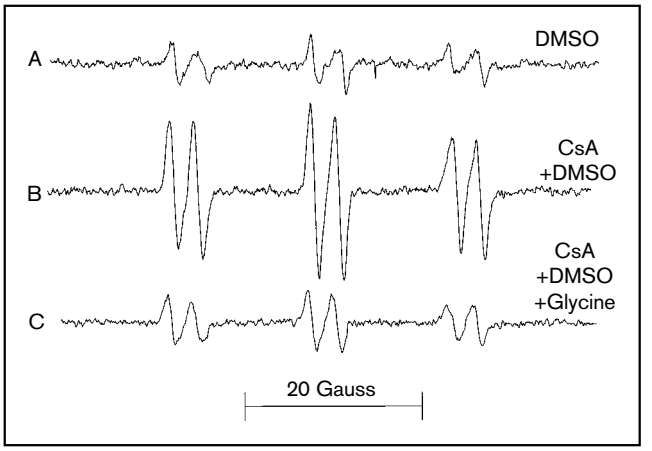
Glycine may exert different effects to prevent ROS formation. In cyclosporin A-induced nephrotoxicity, glycine decreases free radical formation in the kidney (Fig. 3), most likely by inhibiting cyclosporin A-induced increases in renal nerve activity, vasoconstriction and subsequent ischemia/reperfusion [19]. Glycine also appears to suppress ROS formation by inhibiting activation of macrophages. This minimizes subsequent transcription factor activation and cytokine production. For example, dietary glycine prevents hemorrhagic shock-induced activation of Kupffer cells, ameliorates oxidative stress and minimizes the impairment of the activity of antioxidant enzymes (manganese and copper-zinc superoxide dismutase, glutathione peroxidase and catalase) [18,22••]. Polyunsaturated fatty acids and peroxisomal proliferator phthalates promote tumor formation, and this effect is associated with increased free radical formation by Kupffer cells [28–30]. Dietary glycine blocks ROS formation caused by phthalates and corn oil [29,30]. Glycine also decreases superoxide formation in PG-PS-activated splenic macrophages, FMLP-stimulated neutrophils and lipopolysaccharide-stimulated alveolar macrophages [6••,7].

Information on the effects of glycine on production of reactive nitrogen species is limited. In hemorrhagic shock, expression of inducible nitric oxide synthase (NOS) and formation of nitric oxide increase. Dietary glycine blocks these effects [22••]. In contrast, glycine infusion increases renal blood flow and increases glomerular filtration rates in normal rats, an effect that is blocked by L-nitro-L-arginine methyl ester, a pan-NOS inhibitor. Glycine-induced increases in renal blood flow are probably mediated by elevated nitric oxide production [31•].

Transcription factors such as nuclear factor κB and activating protein-1 play a central role in regulating inflammatory processes. Nuclear factor κB regulates inflammation through the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as cyclooxygenase 2 and inducible NOS [32]. ROS activate these transcription factors to induce synthesis of proinflammatory cytokines. Glycine inhibits activation of nuclear factor κB in several models, including hemorrhagic and shock/resuscitation in rats [22••], isolated splenic macrophages exposed to PG-PS [6••], and Kupffer cells from livers exposed to the peroxisomal proliferator WY-14,643 or polyunsaturated fatty acids [29,30].

Release of mediators and the recruitment of circulating leukocytes mediate the inflammatory response. Proinflammatory cytokines play an important role in the progression of the inflammatory process [33•]. Several reports show that glycine suppresses formation of inflammatory cytokines. Endotoxin alone and a combination of ischemia/reperfusion and endotoxin increase TNFα production by Kupffer cells and alveolar macrophages [5,7,18], and glycine largely prevents these effects. Dietary glycine also blocks increases in serum TNFα after hemorrhagic shock [22••] and after experimental sepsis caused by cecal ligation and puncture in rats [34••]. In rats subjected to intestinal ischemia/reperfusion and endotoxin challenge, glycine blocks the early systemic increase of the proinflammatory cytokines TNFα and interleukin-6 and the secretion of anti-inflammatory cytokine interleukin-10 [35•]. Another study found that glycine decreases TNF-α and interleukin-1β but increases interleukin-10 expression in

Figure 3. Dietary glycine prevents cyclosporin A-induced free radical formation



Rats were fed a semi-synthetic powdered diet containing 5% glycine and 15% casein (glycine diet) or 20% casein (control diet) starting 3 days prior to cyclosporin A (CsA) treatment. CsA (25 mg/kg) or an equal volume of vehicle was given to the rat by gavage daily for 6 days. The spin-trapping reagent α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (4-POBN, 1 g/kg) was injected slowly into the tail vein 3 h after the last dose of CsA. Free radical adducts in urine were detected using a Bruker ESP 106 ESR spectrometer. Shown are typical spectra. (A) Free radical adducts were barely detectable in urine from rats treated with dimethyl sulfoxide (DMSO) but (B) increased about four-fold after treatment with CsA/DMSO. (C) Dietary glycine blocked free radical formation caused by CsA. Adapted with permission [19].

monocytes [36]. Glycine also prevents peroxisome proliferator WY-14,643-stimulated hepatocyte proliferation by inhibition of Kupffer cell release of TNFα [30]. In addition, glycine blocks increases in TNFα messenger RNA in ankle homogenates from PG-PS-treated rats [6••]. Whether glycine modifies interleukin-2 production is unclear. Glycine inhibits interleukin-2 production in a mixed mouse lymphocyte culture but has no effect on interleukin-2 production in a mixed rat lymphocyte culture [37,38]. Nonetheless, glycine inhibits the interleukin-2-stimulated proliferation of rat splenic lymphocytes [37].

Lipid-derived mediators such as prostaglandins play an important role in regulating metabolism, microcirculation and inflammation. Glycine inhibits phospholipase A_2 activation caused by hypoxia in renal tubular cells and hepatocytes [39], decreases arachidonic acid release and protects against cell injury. Arachidonic acid can be metabolized to vasoconstrictive and chemotactic eicosanoids, mediators that play important roles in inflammatory process. Many studies show that glycine improves microcirculation, thus preventing tissue hypoxia [19,20••,40]. In some cases, improvement of microcirculation is partially due to the effect of glycine as an inhibitory neurotransmitter [19]. However, inhibition of formation of vasoactive mediators likely also plays an important role. For example, glycine improves microcirculation in models in which nerves are severed, such as in isolated perfused livers and liver grafts after transplantation [40]. Indeed, glycine blocks increases in the chemoattractant leukotriene B_4 after intestinal ischemia/reperfusion [41]. A recent study [42••] showed that Kupffer cells isolated from rats chronically given cyclosporin A produced more PGE_2, and that this effect was blocked by glycine.

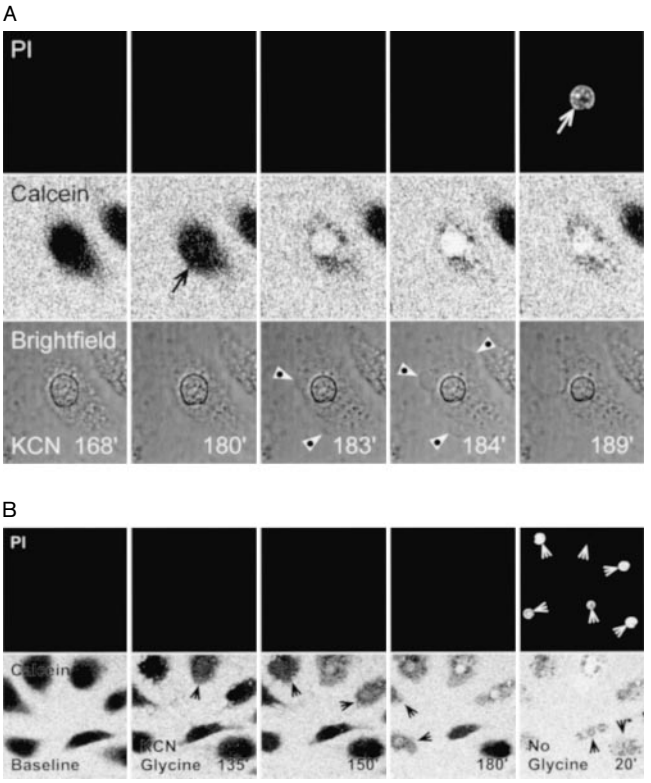
Glycine also inhibits activation of degradative enzymes, such as proteases, thus protecting against cell injury. For example, glycine inhibits calpain, a calcium-dependent protease that is involved in cell injury in ATP-depleted cells [43]. Prevention of phospholipid degradation by inhibition of phospholipase A_2 may also contribute to protection of glycine on cell membrane integrity [44].

Prevention of cell swelling

Glycine strongly protects renal tubular cells, hepatocytes and endothelial cells against injury from hypoxia, ischemia/reperfusion and ATP depletion [43,45–49]. Direct protection of these cells may minimize subsequent inflammatory responses, thus blocking a vicious cycle. How glycine acts directly is not completely clear. Most studies show that glycine protects plasma membrane integrity but does not restore ATP levels or affect intracellular pH [46,48–51]. In contrast, other authors found that glycine restored ATP levels in perfused livers subjected to hypoxia-reoxygenation [21].

In ATP-depleted cells, glycine may act by preventing cellular swelling driven by colloid osmotic gradients [44,52,53••]. During hypoxia, depletion of ATP inhibits the sodium-potassium-adenosine triphosphatase and opens monocation channels [53••]. Although glycine is well known to activate chloride channels via GlyR, in ATP-depleted cells glycine may act antagonistically to block chloride conductance [54]. Several studies showed that glycine blocks hypoxia-induced sodium and chloride entry [21,52,55]. A recent study suggested that late in the progression of ATP depletion injury, a relatively unspecific organic anion channel or 'glycine-sensitive death channel' opens, which initiates a metastable state of accelerated bleb formation and cellular swelling, which culminates in membrane rupture. Two to 3 h after metabolic inhibition with cyanide (chemical hypoxia), small anionic fluorophores like calcein and lucifer yellow abruptly begin to enter hepatic sinusoidal endothelial cells (Fig. 4). Anion entry initiates bleb formation and cell swelling that culminates in permeation of very large molecule weight dextrans (2000 kDa) and cations like propidium. Glycine inhibits this organic anion channel, blocks the metastable period of acceler-

Figure 4. Inhibition by glycine of plasma membrane permeability changes during ATP depletion



Cultured rat hepatic sinusoidal endothelial cells were incubated with cyanide to deplete ATP and incubated with propidium and calcein. (A) Cells excluded red-fluorescing propidium (upper panels) and green-fluorescing calcein (middle panels) after 168 min. No blebbing was apparent in the bright field image (lower panels). After 180 min, calcein began to permeate a cell (middle panel, black arrow). Calcein permeation continued and was essentially complete after 184 min. Bleb formation and cellular swelling became observable after 183 min and obvious after 184 min (dotted arrowheads). Bleb growth continued to 189 min. After this bleb growth, propidium labeled the nucleus after 189 min (white arrow). (B) Cells also began to fill with calcein relatively late during chemical hypoxia in the presence of 3 mM glycine (lower panels, black arrows). In the presence of glycine, calcein took at least 45 min to equilibrate with the intracellular space (180′, lower panel), which was much slower than in the absence of glycine. Subsequently, when glycine was removed, all nuclei labeled with propidium (no glycine, upper panels, white arrows), and cells that had not previously taken up calcein did so (black arrows). Adapted with permission [53••].

ated cellular swelling and prevents uptake of high molecular weight dextrans and propidium [53••]. These results are consistent with the conclusion that rapid swelling during the metastable state is due to accelerated sodium chloride entry when glycine-sensitive channels open to conduct chloride (and other low molecular weight anions) in the presence of already open monovalent cation channels to conduct sodium. Swelling during the metastable state is driven by the oncotic (colloid osmotic) pressure of macromolecules in the cytosol. Continued swelling leads to membrane stretching and ultimately bursting of the plasma membrane (Fig. 5). The structure and molecular identity of the glycine-sensitive death channel remain unknown, and further work is needed to isolate and characterize this channel.

Other studies showed that glycine prevents entry of sodium and the non-physiological cations cobalt and nickel into hypoxic hepatocytes and indicated that the protective effect of glycine does not require the presence of chloride, suggesting that glycine protects against hypoxic injury of hepatocytes by inhibition of ion flux through relatively unspecific leaks [50]. This work is in contrast to the result reported by Carini and coworkers [52]. In ATP depletion models, strychnine is just as protective as glycine [54]. Moreover, cell-impermeable strychnine-fluorescein conjugates also protect [56••], which is consistent with the conclusion that the strychnine and glycine act at the outer surface of plasma membranes to mediate cytoprotection. This study also supported the hypothesis that porous defects in the plasma membrane develop in ATP-depleted cells and that glycine and strychnine derivatives protect by low affinity interactions with a multimeric channel proteins [56••].

Taken together, glycine works by direct protection of the plasma membranes of ATP-depleted cells and inhibition of inflammatory and immune processes. The exact mechanism of glycine protection must be determined for a particular circumstance, and mechanisms will likely vary in different disease states. Moreover, by acting at different levels, glycine may exert multiple synergistic protective effects.

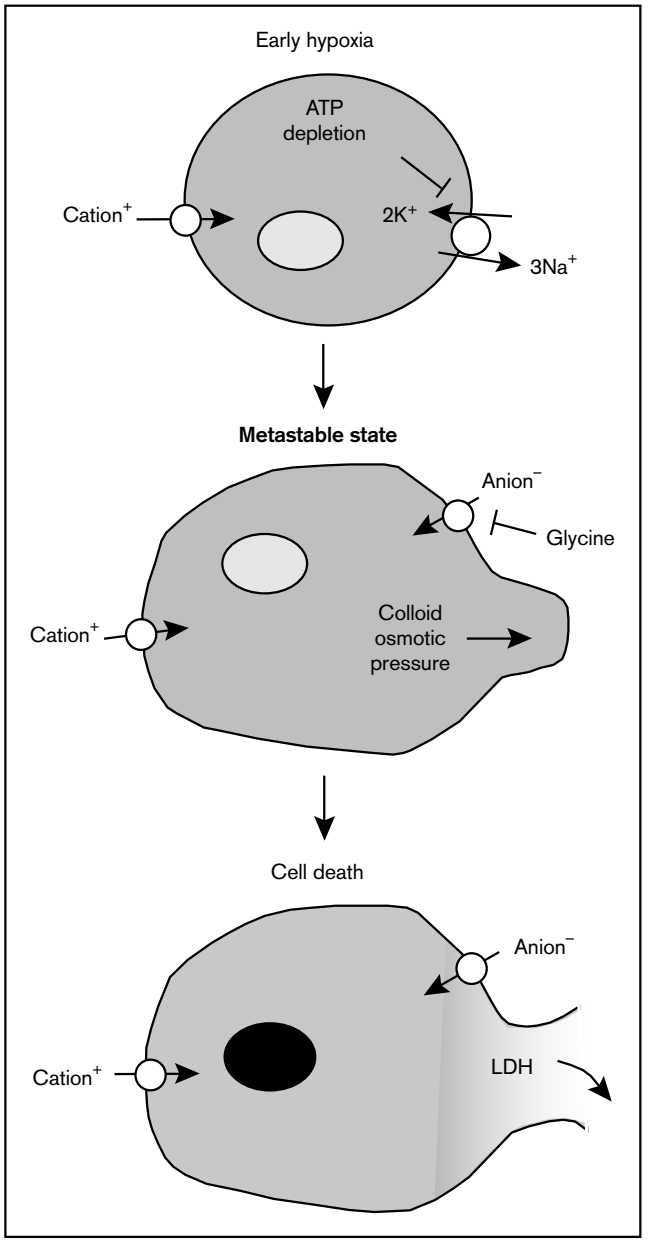
Examples of protection by glycine in disease status

Regardless of controversies on mechanisms, the beneficial effects of glycine in a variety of diseases are well documented. Here, some of these protective effects are reviewed.

Experimental ischemia–reperfusion injury

Ischemia–reperfusion injury occurs in trauma, hemorrhagic shock, cardiovascular disease, stroke and following major surgery, including tumor resection and transplantation. Drugs or chemicals that cause vasoconstriction or disturb mitochondrial function can also cause ischemia or ischemia-like injury. Decreased ATP synthesis during hypoxia and ischemia leads to cell death. Glycine can directly protect cells from hypoxic injury, as described above. Although ischemia damages tissues, reperfusion of ischemic tissue causes further injury. Activation of macrophages and neutrophils, production of free radicals and toxic mediators and activation of degradative enzymes such as proteases lead to an inflammatory

Figure 5. Proposed mechanism of plasma membrane permeability changes leading to cell death during hypoxia: role of a glycine-sensitive death channel



During early hypoxia, sodium and potassium gradients collapse due to ATP depletion, inhibition of the sodium-potassium-adenosine triphosphatase and opening of monocation channels. Little cell swelling occurs at this stage as plasma membranes remain impermeable to anions. Late in hypoxia, a glycine-sensitive anion channel (death channel) opens abruptly, permitting anions including chloride to enter. As cation channels are already open, anion channel opening initiates a metastable state characterized by accelerated cell surface bleb formation and cellular swelling driven by colloid osmotic pressure gradients. Swelling finally causes cell membrane rupture and cell death. At this time, propidium, a cell viability indicator, enters to label the nucleus. LDH, lactate dehydrogenase. Adapted with permission [53••].

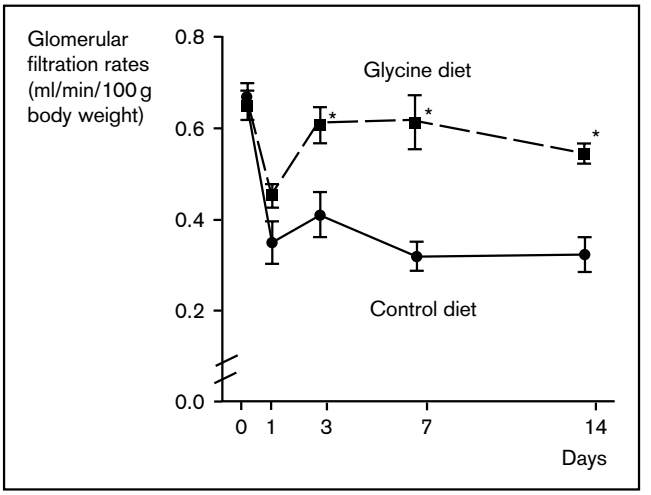
response, tissue damage and multiple organ failure [23,24,57]. Glycine suppresses these events by mechanisms described above, thus protecting against ischemia–reperfusion injury.

Glycine has beneficial effects in several experimental hypoxia-reperfusion models. For instance, in isolated liver perfusion and *in vivo* warm ischemia/reperfusion, glycine decreases enzyme release (lactate dehydrogenase, alanine aminotransferase and aspartate aminotransferase), improves liver function, and improves microcirculation after reperfusion [21,58]. A recent study [20••] showed that dietary glycine (5%) is also protective *in vivo* against renal ischemia (cross-clamping the left renal vessels for 15 min) followed by reperfusion. With glycine treatment, pathological changes such as tubular injury, cast formation, leukocyte infiltration, interstitial fibrosis, and urinary lactate dehydrogenase release are minimized by glycine. Post-ischemia/reperfusion glomerular filtration rates recover better and more quickly (Fig. 6). These results indicate that administration of glycine decreases ischemia–reperfusion injury in the kidney *in vivo*. Importantly, glycine not only prevents early injury but also minimizes chronic hypoxia and fibrosis after ischemia/reperfusion [20••]. However, in more severe hypoxia/reperfusion injury in the kidney, glycine may not be potent enough to provide overt protection [59].

In the intestine, glycine is considered biologically neutral and is often used as an isonitrogenous control in studies on the effects of glutamine. However, a recent study [60••] showed protective effects of glycine in ischemia–reperfusion injury to the intestine. Infusion with 20% glycine also increases mucosal protein and deoxyribonucleic acid content, decreases intestinal myeloperoxidase activity, and maintains mucosal glutaminase activity [60••]. These results are particularly relevant to the nutritional care of critically ill patients, because ischemia–reperfusion injury is a putative mechanism underlying the loss of gut barrier in these patients.

Glycine also protects skeletal muscle subjected to ischemia–reperfusion injury [61••]. Perfusion of 2.2% glycine for 15 min decreases muscular edema and necrosis, increases ATP content and preserves muscle function [61••]. A recent study from our laboratory showed that glycine diminishes pathological changes and creatine kinase release after coronary artery occlusion/reperfusion in rats, and this effect is associated with decreased free radical formation (C.A. Weigel *et al.*, personal communication). Taken together, these data suggest that glycine is a promising therapy for injury to a variety of organs or tissue caused by ischemia/reperfusion.

Figure 6. Dietary glycine improves kidney function after renal ischemia/reperfusion



The left kidney was subjected to 15 min of ischemia, and the right kidney was removed. Urine and blood samples were collected, and glomerular filtration rates were determined from creatinine levels in blood and urine, urine volume collected in 24 h and body weight. Values are means ± SE ($n = 8$/group). *$P < 0.05$ versus the control diet group by one-way repeated-measures ANOVA. Adapted with permission [20••].

Transplantation

Ischemia–reperfusion injury associated with cold ischemic storage of organs for transplantation surgery plays an important role in primary graft failure. Thus, glycine is a candidate as therapy to prevent primary graft failure. Glycine diminishes hypoxic and cold ischemic injury to dog and rabbit kidneys and improves graft function after transplantation [62]. In addition, glycine-containing Carolina rinse solution protects kidneys against storage/reperfusion injury and improves renal graft function and survival after kidney transplantation [63•]. The use of glycine in transplantation is most widely studied in liver transplantation. Glycine addition to cold storage solution and to Carolina rinse solution for rinsing grafts at the end of cold storage reduces reperfusion injury, improves graft function, and increases graft survival after rat liver transplantation, most likely by decreasing non-parenchymal cell injury [64,65]. Glycine treatment of liver transplant recipients also improves the outcome. Organ manipulation during harvest of livers causes disturbance of microcirculation and hypoxia, which decreases graft survival after implantation [40,66••]. Intravenous infusion of glycine to donor rats significantly improves graft survival [40,66••]. Due to a severe shortage of donor organs, non-heart-beating donors are gaining increasing importance as a potential source of transplantable organs for clinical use. In grafts from non-heart-beating porcine donors, glycine (25 mg/kg continuous perfusion during normothermic recirculation) diminishes reperfusion injury to parenchymal and endothelial cells after transplantation [67•,68]. Importantly, glycine markedly reduces reperfusion injury in human liver transplantation, as shown in a recent clinical trial [69••]. Recipients were infused intravenously with 250 ml of 300 mM glycine for 1 h before implantation and 25 ml once daily after transplantation. Peak transaminase levels after transplantation decreased three to four fold, and total bilirubin decreased significantly [69••]. These results suggest that glycine is an effective therapy to prevent liver graft injury in a clinical setting. As the number of patients in this study was limited, a large-scale clinical trial is necessary to evaluate its effect after human liver transplantation. Glycine also protects intestine grafts after cold storage for 48 h [41]. Glycine improves blood flow and oxygen supply, decreases neutrophil infiltration and attenuates pathological changes such as loss of villus epithelium, decreased villus height, and venous congestion [41].

Rejection is another important factor decreasing graft survival. Glycine appears to moderate immunological reactions and may prove useful in diminishing rejection after transplantation. High doses of glycine (50–300 mg/kg) administered daily for 10 days in rabbits challenged with typhoid 'H' antigen and sheep erythrocyte antigen cause a dose-dependent decrease of antibody titer [70]. Glycine also inhibits cell proliferation on mixed lymphocyte culture by 60% [9]. These results indicate that glycine is a potential immunomodulating agent. Consistent with this hypothesis, dietary glycine inhibits chronic rejection of aortic allografts [16]. A glycine diet minimizes intimal thickening and medial thinning and decreases leukocyte infiltration in the adventitia of allografts. Glycine also inhibits proliferation and migration of aortic smooth muscle cells in culture by 45 and 60%, respectively. These results indicate that dietary glycine minimizes histopathological changes of chronic rejection by reducing the immune response and, in part, by minimizing proliferation and migration of smooth muscle cells [16]. In skin transplantation from F344 to Lewis rats (minor histocompatibility complex barrier), dietary glycine applied with low dose cyclosporin A prolongs survival of allografts compared with low dose cyclosporin A alone [37]. Similarly, in kidney transplantation from DA to Lewis rats (major histocompatibility complex barrier), dietary glycine applied with low dose cyclosporin A prolongs survival of allografts and markedly improves renal function compared with low dose cyclosporin A alone [37]. However, glycine alone does not prolong graft survival. These results indicate that glycine has moderate immunosuppressive effects.

Interestingly, glycine also has protective effects on gel-entrapped rat hepatocytes in a bioartificial liver [71]. Glycine inhibits cell necrosis after exposure to anoxia, and the maximal protective effect was achieved at 3 mM of glycine [71].

Shock

Hemorrhagic and endotoxic shock frequently occur in critically ill patients. Among others, hypoxia/reperfusion, activation of inflammatory cells, release of toxic mediators and disturbance in coagulation play important roles in the occurrence of multiple organ failure caused by shock [57]. Glycine has a potent inhibitory effect on these events, as mentioned above, and therefore could be an effective therapy for shock.

Glycine decreases organ injury (Fig. 7) and improves survival after hemorrhage shock/resuscitation in a dose-dependent manner [18]. Another study also showed that dietary glycine significantly reduces mortality, decreases transaminase release and diminishes hepatic necrosis after hemorrhage shock/resuscitation [22••]. Short-term glycine treatment almost totally prevents mortality and decreases serum transaminase levels, hepatic necrosis, and lung injury caused by endotoxin treatment and endotoxin with ischemia/reperfusion in rodents [5,7,35•]. Chronic glycine treatment (4 weeks) improves survival and decreases inflammation after endotoxin but does not improve liver pathology [7]. The selective efficacy after chronic glycine treatment may be due to downregulation of glycine-gated chloride channels on Kupffer cells but not on alveolar macrophages and neutrophils [7]. Thus, chronic glycine may improve survival by decreasing lung inflammation. Glycine also attenuates liver injury, improves liver function and totally prevents mortality in experimental sepsis caused by cecal ligation and puncture [34••]. Clearly, glycine provides potent protection on hemorrhagic, septic and endotoxin shock. It is unclear, however, if glycine minimizes shock caused by gram-positive bacteria.

Liver fibrosis

Hepatic injury and the subsequent repair and inflammatory processes augment fibrosis [72]. Kupffer cells release many mediators that activate stellate cells, including TNFα, transforming growth factor-β, hepatocyte growth factor, platelet-derived growth factor and ROS [72]. Glycine, which protects against cell injury, inhibits activation of Kupffer cells and production of proinflammatory cytokines, might therefore be expected to ameliorate liver fibrosis. In confirmation, glycine attenuates liver fibrosis caused by carbon tetrachloride [73••]. Attenuation of fibrosis is associated with decreased collagen synthesis rather than increased collagen degradation. Glycine inhibits activation of stellate cells and synthesis of TGFβ and these effects can be mimicked by removal of Kupffer cells with gadolinium chloride [73••]. Therefore, glycine likely works by inhibiting Kupffer cells activation. In Wistar rats treated with 'complex pathogens', dietary glycine attenuates the extent of liver fibrosis [74•], an effect associated with inhibition of CD_{14} expression and TNFα synthesis [74•]. It is unclear, however, what these complex pathogens are. In contrast, in experimental cholestasis caused by bile duct ligation, glycine decreased hepatocyte necrosis but did not block liver fibrosis [14]. Interestingly, gadolinium chloride, which selectively destroys Kupffer cells, likewise does not prevent fibrosis [14], indicating that Kupffer cells do not play an important role in cholestasis-induced fibrosis. Thus, the effects of glycine appear to depend on the properties of the stimulus.

Alcoholic liver disease

Glycine diminished alcohol-induced transaminase release, liver steatosis and necrosis in a Tsukamoto–French model of intragastric alcohol feeding [75]. Glycine accelerates the first-pass elimination of alcohol by the stomach thus preventing alcohol from reaching the liver [75]. After induction of alcoholic hepatitis, dietary glycine accelerates recovery from injury. This effect appears related to decreased TNFα synthesis [76].

Gastric ulcer

Glycine also dose-dependently decreases acid secretion caused by pylorus-ligation [77] and protects against experimental gastric lesions induced by hypothermic-restraint stress, indomethacin and necrotizing agents including 80% ethanol, 0.2 M sodium hydroxide and 0.6 M hydrochloric acid in rats. Thus, glycine possesses significant anti-ulcer and cytoprotective activity [77]. However, further studies are needed to elucidate the mechanisms of glycine's action on the stomach, and to determine its role in the prophylaxis and treatment of gastric ulcer disease.

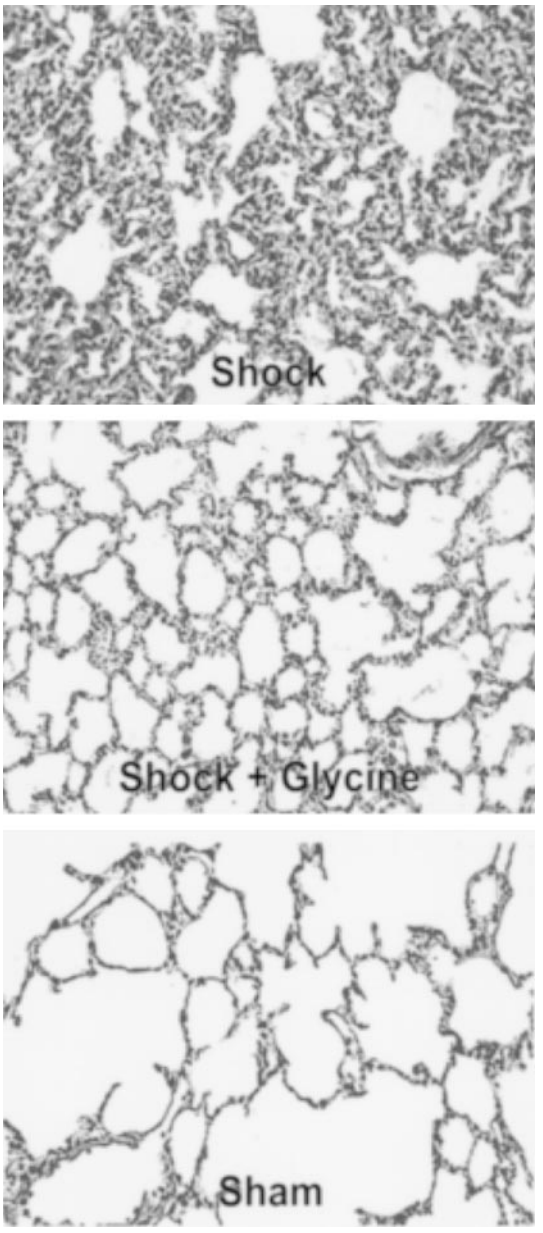
Arthritis

As glycine is a potential immunomodulator that inhibits inflammation, its effect on arthritis is studied in a PG-PS model of arthritis *in vivo*. PG-PS is a primary structural component of gram-positive bacterial cell walls and causes rheumatoid-like arthritis in rats. In rats injected with PG-PS intra-articularly, dietary glycine attenuates ankle swelling and decreases infiltration of inflammatory cells, edema, and synovial hyperplasia in the joint [6••]. Glycine also blunts increases of TNFα expression in the ankle and inhibits TNFα release from splenic macrophages. This work supports the hypothesis that glycine prevents reactive arthritis by blunting cytokine release from macrophages [6••].

Cancer

Peroxisomal proliferators and polyunsaturated fatty acids act as tumor promoters possibly by increasing cell proliferation, and Kupffer cells are rich sources of mitogenic cytokines such as TNFα [28]. Dietary glycine inhibits cell proliferation caused by WY-14,643, a peroxisomal proliferator, and by corn oil [30]. Glycine

Figure 7. Glycine minimizes pathological changes in the lung caused by hemorrhagic shock/resuscitation



Shock was induced by withdrawing blood from the right carotid artery to decrease mean arterial pressure to 30–35 mmHg. After 60 min of hypotension, rats were resuscitated by transfusion of 60% shed blood and lactated Ringer's solution of twice the shed blood volume over 1 h. Glycine (45 mg/kg) was injected intravenously just prior to resuscitation. Organs were perfused with Krebs–Henseleit buffer (pH. 7.4) 18 h after shock to remove blood and fixed with 1% paraformaldehyde. Sections were stained with hematoxylin and eosin. Shown are representative images of at least four sections from each group. Lung specimens from rats receiving saline injection at the end of shock exhibited hemorrhage, edema, increased cellularity in interstitial tissue and massive infiltration of inflammatory cells (upper panel). Glycine minimized these pathological changes (middle panel). Adapted with permission [18].

likely acts by blocking activation of nuclear factor κB and synthesis of TNFα by Kupffer cells [30]. Glycine also inhibits tumor growth of implanted B16 melanoma cells by 65%, indicating a general anticancer property [78].

Drug- or toxicant-induced hepatic and renal injury

Glycine protects against injury to cells or organs such as liver and kidney caused by a wide variety of chemicals, drugs and toxicants [19,47,48,54]. Mechanisms underlying this protection appear to depend on the different types of toxicants, and each cannot be discussed in detail here. Some drugs have multiple toxic effects, such as cyclosporin A. Nephrotoxicity occurs in about 30% of patients receiving cyclosporin A for immunosuppression. Liver injury also occurs. Dietary glycine attenuates both the nephrotoxicity [19] and the hepatotoxicity caused by cyclosporin A [42••]. Cyclosporin A also causes a hypermetabolic state and hypoxia in liver tissue, which inhibits liver function. These effects are diminished by glycine. Such protection is associated with an inhibition of Kupffer cell release of PGE_2 [42••]. Thus, dietary glycine supplementation to patients taking cyclosporin A may be useful to prevent the adverse effects of this widely used immunosuppressive drug. In addition, glycine may augment immunosuppressive effects when used together with cyclosporin A [37].

Toxicity

Glycine is already abundant in the diet as a component released during digestion of protein and is considered to be non-toxic even in high doses. However, two recent reports suggest that glycine might have toxic effects [79,80••]. Cardiac arrest occurred in a 69-year-old man when a glycine irrigation fluid was used during prostatic surgery [80••]. It is not clear, however, if other factors were involved in this rare complication. In an animal study, intravenous infusion of a high dose of glycine (1.5%, 300 ml/kg body weight) induced bradycardia and decreased survival [79]. However, this dose is about 50-fold higher than that used in most studies showing protective effects of glycine in rats [18] or in humans after liver transplantation [69••]. As high-dose glycine may cause toxic effects, further study is needed to investigate the safe dose range and routes of administration.

Conclusion

Glycine has a wide spectrum of protective properties against different diseases and injuries. As such, glycine represents a new antiinflammatory, immunomudulatory and cytoprotective agent. More studies are needed to investigate the effects of the amino acid in humans on diseases where free radicals, proinflammatory cytokines and ischemia/reperfusion are involved. Additionally,

glycine should be explored as a potential protective agent in animal models of burn injury, gram-positive sepsis and cardiovascular disease. Mechanisms of glycine protection remain to be completely elucidated, and caution should be paid to the safe dose and method of administration.

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