

Glycine reduces platelet aggregation

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Abstract It has been demonstrated that a wide variety of white blood cells and macrophages (i.e. Kupffer cells, alveolar and peritoneal macrophages and neutrophils) contain glycine-gated chloride channels. Binding of glycine on the receptor stimulates Cl^- influx causing membrane hyperpolarization that prevents agonist-induced influx of calcium. Since platelet-aggregation is calcium-dependent, this study was designed to test the hypothesis that glycine would inhibit platelet aggregation. Rats were fed diets rich of glycine for 5 days, while controls received isonitrogenous valine. The bleeding time and ADP- and collagen-induced platelet aggregation were measured. Dietary glycine significantly increased bleeding time about twofold compared to valine-treated controls. Furthermore, the amplitude of platelet aggregation stimulated with ADP or

collagen was significantly decreased in whole blood drawn from rats fed 2.5 or 5 % dietary glycine by over 50 %. Addition of glycine in vitro (1–10 mM) also blunted rat platelet aggregation in a dose-dependent manner. Strychnine, a glycine receptor antagonist, abrogated the inhibitory effect of glycine on platelet-aggregation in vitro suggesting the glycine works via a glycine receptor. Glycine also blunted aggregation of human platelets. Further, the glycine receptor was detected in both rat and human platelets by western blotting. Based on these data, it is concluded that glycine prevents aggregation of platelets in a dose-dependent manner via mechanisms involving a glycine receptor.

Keywords ADP · Collagen · $[\text{Ca}^{2+}]_i$ · Glycine receptor

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Introduction

Platelet thrombus formation represents an essential physiologic defense mechanism in primary hemostasis to arrest bleeding at sites of vessel disruption and can contribute to generation of pathologic intra-vessel thrombosis (O'Brien 1964; Zucker 1949). Further, both experimental and clinical observations have demonstrated a major role of platelets in the pathophysiology of thrombotic states and vascular diseases (Becker 1991; Gori 2011; Kaliman et al. 1983) and there is a link between platelet activation, arrhythmogenesis and sudden death (Flores 1996). Moreover, platelets are involved in the pathobiology of myocardial ischemia, infarction and hypertension (Fuster et al. 1992). $[\text{Ca}^{2+}]_i$ plays a central regulatory role in platelet function. In the resting state, platelet $[\text{Ca}^{2+}]_i$ is maintained at low levels (Flores 1996). When $[\text{Ca}^{2+}]_i$ is increased by agonists or ionophores, the platelets become activated and undergo

aggregation (Cronberg and Caen 1970; Jancinova et al. 1992). The resting $[Ca^{2+}]_i$ in platelets has been reported to be higher in hypertensive patients than in normotensive individuals (Bruschi et al. 1985; Cooper et al. 1987; Gulati et al. 1996). Hyperactivity of the Na^+/H^+ exchanger leading to more alkaline intracellular pH has also been described in platelets from hypertensive patients (Poch et al. 1993, 1994; Siffert 1995).

Glycine, a simple non-essential, non-toxic amino acid and inhibitory neurotransmitter, has been shown to hyperpolarize postsynaptic motor neurons by increasing chloride conductance (Curtis and Duggan 1969; Curtis et al. 1968a, b). Glycine exerts its inhibitory actions by binding its receptor (GlyR) which is largely localized in postsynaptic neuronal membranes (Werman et al. 1967) and is often referred to as a glycine-gated chloride channel. This effect of glycine was shown to be selectively blocked by strychnine, a plant alkaloid (Young and Snyder 1973). We demonstrated that a wide variety of white blood cells and macrophages (i.e., Kupffer cells, alveolar and peritoneal macrophages and neutrophils) also contain glycine-gated chloride channels (Ikejima et al. 1997; Wheeler et al. 1999, 2000). Binding of glycine on the receptor stimulates Cl^- influx causing membrane hyperpolarization that prevents agonist-induced influx of calcium.

Since platelet-aggregation is calcium-dependent, this study was designed to test the hypothesis that platelets possess a glycine-gated chloride channel and that glycine could modulate platelet aggregation.

Materials and methods

Experimental animals and treatment

Female Sprague-Dawley rats (250–300 g) were allowed free access to standard laboratory rat chow (Agaway PROLAB RMH 3000, Syracuse NY) and tap water. Groups were fed 0.313, 0.625, 1.25, 2.5, and 5 % dietary glycine for 5 days while controls were given isonitrogenous valine. All procedures were performed on ether anaesthetized rats, and animals were given humane care in compliance with institutional guidelines.

Bleeding time, blood ionic calcium levels and pH

The bleeding time was determined by transection of the tail 5 mm from the tip. The tail was immersed in isotonic saline at room temperature as described elsewhere (Dejana et al. 1982) and bleeding time was recorded. Blood ionic calcium and pH were analyzed with ion-selective electrode techniques using a Blood Gas Analyzer (Corning 288).

Platelet aggregation

Heparinized blood (10 IU/ml) was collected from the vena cava of rats under anesthesia with ether. For whole blood aggregation studies, 0.5 ml blood was diluted with normal saline to 1 ml. Some samples were preincubated with glycine at a final concentration of 1.3 or 10 mM at 37 °C for 10 min. The sample was stirred continuously at 37 °C with a Teflon coated stir-bar (1,000 rpm), the electrode was placed in the sample and the aggregometer (Chronolog Corp., Havertown, PA, USA) was calibrated. Subsequently, stimuli (collagen 8 µg/ml or ADP 0.3 µM) were added and changes in the impedance were recorded.

Human blood samples were collected into 3.15 % trisodium citrate (9:1 v/v) after puncture of the cubital vein. Platelet-rich plasma was obtained by centrifugation at $1,000\times g$ for 20 min, and platelet-poor plasma was obtained by further centrifugation of the platelet-rich plasma ($1,000\times g$, 10 min), and was used to calibrate the aggregometer as 100 % transmission. Platelet-rich plasma was equilibrated at 37 °C for 1 min under constant stirring (1,000 rpm) before aggregation was induced. Final concentration of collagen (bovine type 3, acid soluble; Sigma, St. Louis, MO, USA) used to induce aggregation was 8 µg/ml. Aggregation was monitored as changes in light transmission.

Isolation of platelets from total blood

Human blood (3 ml) was collected in citrate tubes (SARSTEDT, Germany) and centrifuged ($200\times g$, 10 min, RT). The supernatant (350 µl) was incubated (10 min, RT) with 50 µl CD45 MicroBeads and 50 µl Glycophorin A MicroBeads (Miltenyi, Germany). The MS Column (Miltenyi, Germany) was loaded with the supernatant and washed with 500 µl PBS. The unlabeled platelets passed through and were collected. The column was removed from the magnet and washed with 500 µl PBS to release the erythrocytes.

Western blotting

Cells were centrifuged (12,000 rpm, 20 min, 4 °C) and resuspended in 100 µl RIPA Buffer (1% Triton X-100), containing complete protease inhibitor cocktail (Roche, Germany). Protein lysates (10 µg) were separated on a 10 % SDS-PAGE gel, transferred to a nitrocellulose membrane (Bio-Rad, Germany), blocked in 5 % non-fat dried milk, incubated with primary antibody and visualized with appropriate horseradish peroxidase-coupled secondary antibodies (Santa Cruz) using enhanced chemiluminescence (ECL; Amersham). Human anti-glycine receptor alpha 1 + alpha 2 antibody was from Abcam (Cambridge, UK), rat anti-GlyR4a monoclonal primary antibody was

from Cederlane (QC, Canada), anti β -actin antibody was from Sigma-Aldrich (St. Louis, USA).

Statistics

Mean values $\pm$ SEM for groups were compared using Student's *t* test or analysis of variance (one-way ANOVA) with Student–Newman–Keul's post hoc test as appropriate. $p < 0.05$ was selected prior to the study as the criterion for significance.

Results

Glycine increases bleeding time in rats

Bleeding time was increased more than twofold in rats after 5 % dietary glycine for 5 days compared to rats fed isonitrogenous valine ($p < 0.05$) (Fig. 1). In contrast, calcium levels and pH in the blood were not altered by dietary glycine (data not shown), indicating that these factors are not involved in changes of bleeding time.

Glycine in vivo prevents platelet aggregation in rats

Heparinized blood was drawn from rats fed diets containing 0.6, 1.3, 2.5, and 5 % glycine for 5 days. Naive rats and

control rats fed isonitrogenous valine displayed amplitude changes around 20 Ω after ADP or collagen addition; however, dietary glycine decreased aggregation in a dose-dependent manner (Fig. 2a, b). Dietary glycine at concentrations of 2.5 and 5 % given for 5 days significantly decreased the amplitude of platelet aggregation over 50 % (Fig. 2).

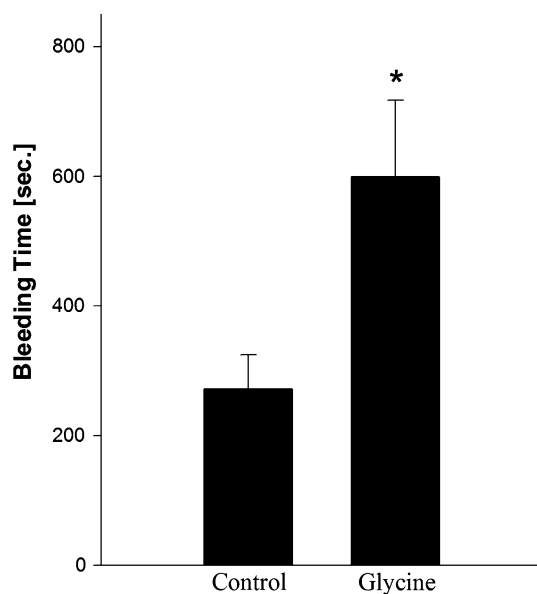


Fig. 1 The effect of glycine on bleeding time in rats. Rats were fed 5 % dietary glycine for 5 days while controls received a diet containing 5 % isonitrogenous valine. Bleeding time was measured as described in “Materials and methods”. Values are mean $\pm$ SEM, $n = 6$. * $p < 0.05$ compared to valine treated controls using Student's *t* test

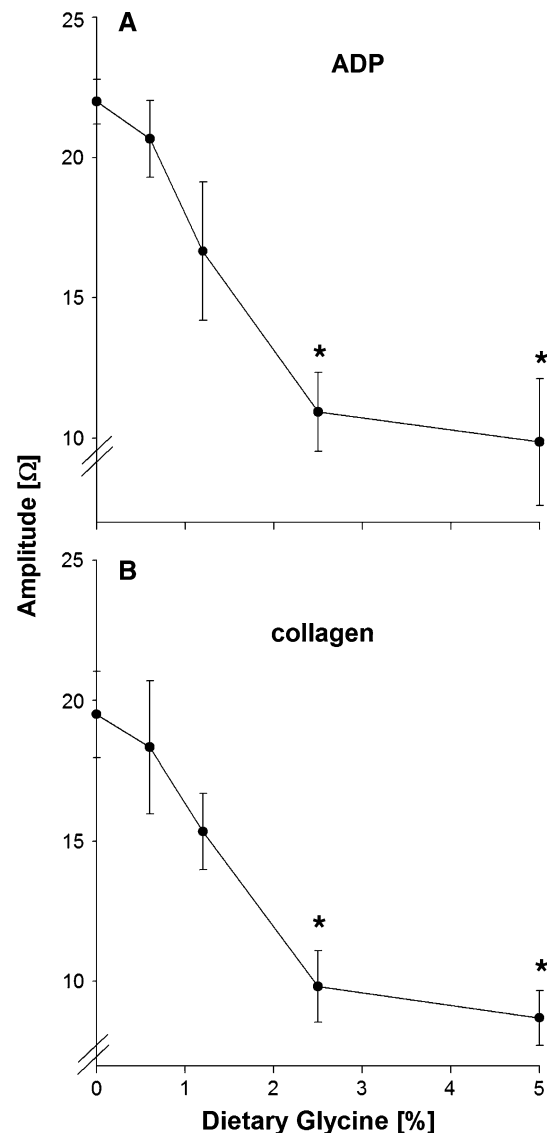


Fig. 2 Dietary glycine inhibits rat platelet aggregation in vivo in a dose-dependent manner. Blood was drawn from rats fed diets containing 0.6, 1.3, 2.5 or 5 % glycine for 5 days. Heparinized whole blood was used to measure the amplitude of platelet aggregation after **a** ADP (0.3 μ M) or **b** collagen (8 μ g/ml) from changes of impedance at 37 $^{\circ}$ C with an aggregometer as described in “Materials and methods”. Values are mean $\pm$ SEM, $n = 6$, $p < 0.05$ for comparison to platelet aggregation without glycine (control) by one-way ANOVA with Student–Newman–Keul's post hoc test

Rat platelet aggregation is reduced by glycine in vitro

Platelet function was measured in heparinized blood by the change of impedance due to platelet aggregation using ADP or collagen as agonists. In controls, the change in amplitude was about 21 Ω after addition of either ADP or collagen. Glycine, at final concentrations from 1 to 10 mM, reduced platelet aggregation in a dose-dependent manner (Fig. 3). The amplitude of aggregation after ADP was decreased about 40 % by glycine at concentrations of 3 and 10 mM, respectively (Fig. 3a). When collagen was used as agonist similar results were obtained (Fig. 3b).

Strychnine blunts inhibitory effects of glycine on rat platelet aggregation

To investigate if glycine receptors are involved in the decreases of platelet aggregation, effects of strychnine (1 μ M), a specific glycine receptor antagonist (Ikejima et al. 1997), were evaluated. Controls had values of $20.6 \pm 0.5 \Omega$, which were reduced by glycine to $15.4 \pm 0.5 \Omega$ ($p < 0.05$) (Fig. 4). This effect of glycine was largely blocked with strychnine ($p < 0.05$).

Human platelet aggregation is reduced by glycine in vitro

Effects of glycine on human platelet aggregation were also studied in platelet-rich plasma from healthy donors. While normal aggregation was observed in the absence of glycine, the amplitude of platelet aggregation was dramatically decreased by glycine (data not shown). Glycine at final concentrations of 3 and 10 mM significantly decreased the amplitude of aggregation of human platelets stimulated with ADP from control values 31–25 and 22 Ω , respectively.

Human and rat platelets express a glycine receptor

The glycine receptor 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 (Pfeiffer et al. 1984). In purified spinal cord membranes, the 48 kDa α -subunit of the glycine receptor was detected by western blotting (Fig. 5). The α -subunit was also detected in purified platelet membranes from rats (Fig. 5), indicating that platelets also contain glycine receptor. To confirm this finding, we isolated platelets and erythrocytes from human blood, using CD45 and Glycophorin A MicroBeads. As expected, the glycine receptor was detected in human platelets but not in erythrocytes (Fig. 6).

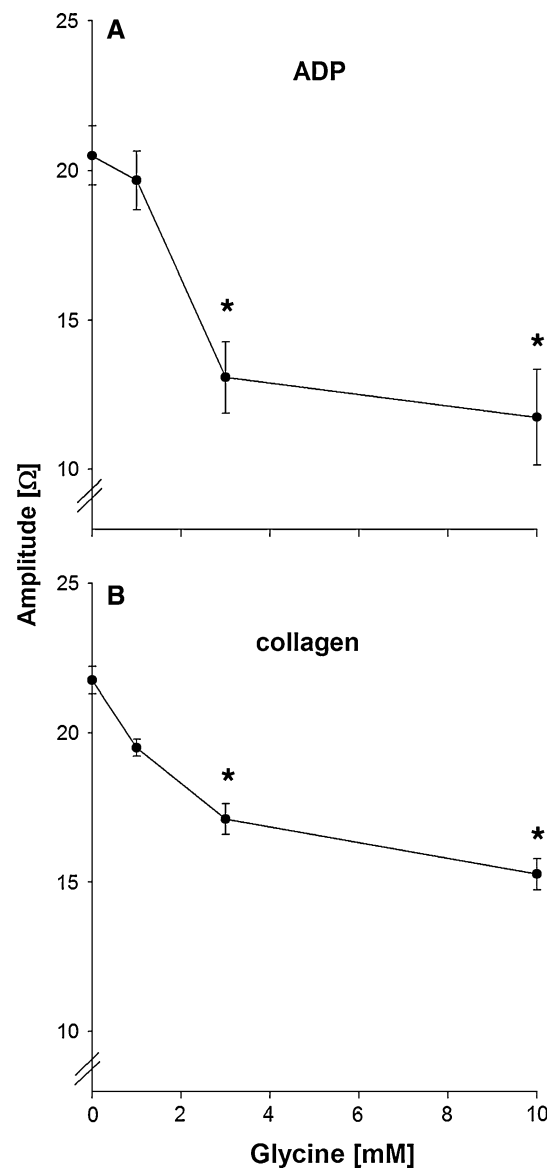


Fig. 3 Glycine inhibits rat platelet aggregation in vitro in a dose-dependent manner. Heparinized blood was incubated at 37 °C for 10 min with glycine at a final concentration of 1 to 10 mM. Subsequently, platelet aggregation was induced with either **a** ADP (0.3 μ M) or **b** collagen (8 μ g/ml), and changes of impedance were measured as described in “Materials and methods”. Values are mean $\pm$ SEM, $n = 6$, $p < 0.05$ for comparison to platelet aggregation without glycine (control) by one-way ANOVA with Student–Newman–Keul’s post hoc test

Discussion

Inhibition of platelet aggregation with glycine: a novel strategy to prevent cardiovascular disease

Over the last two decades, mortality from coronary heart disease and cerebrovascular disease declined by about 30 % in the EU. Nevertheless, cardiovascular disease remains the leading cause of death in Europe (Bertuccio

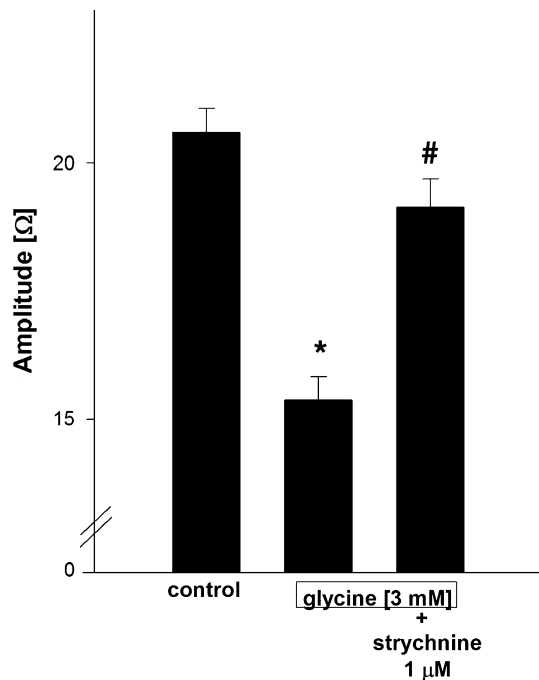


Fig. 4 Strychnine blunts inhibitory effects of glycine on rat platelet aggregation. Heparinized whole blood was incubated at 37 °C for 10 min with glycine at a final concentration of 3 mM. Subsequently, platelet aggregation was induced with collagen (8 μg/ml) and changes of impedance were measured as described in “Materials and methods”. Strychnine was added at a final concentration of 1 μM. Values are mean ± SEM, $n = 8$. * $p < 0.05$ for comparison to the control group; # $p < 0.05$ for comparison to the glycine group by one-way ANOVA with Student–Newman–Keul’s post hoc test

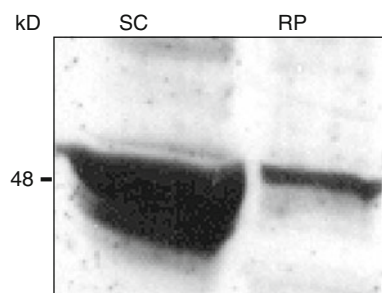


Fig. 5 Western blot of glycine receptor in spinal cord and rat platelets. Western blotting was performed as described in “Materials and methods”. Western blot of 3 μg spinal cord membrane extract (SC) and 30 μg of platelet membrane extract (RP) using anti-GlyR4a monoclonal antibody is shown

et al. 2011). During 2012, approximately 1.1 million persons are expected to have a coronary heart disease event, and prevention remains the key strategy for reducing the mortality (Groseclose 2001). Excessive platelet accumulation and recruitment lead to vessel narrowing and occlusion has been considered as one of the factors leading to cardiovascular disease, stroke and sudden death (Flores 1996; Fuster et al. 1992). A number of therapeutic reagents,

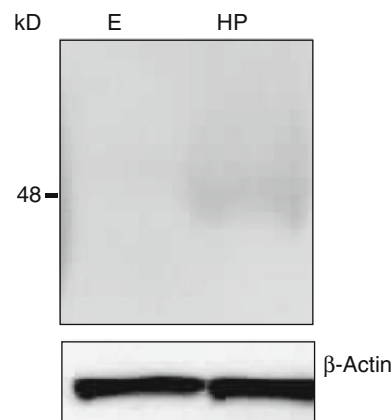


Fig. 6 Western blot of glycine receptor in erythrocytes and human platelets. Western blotting was performed as described in “Materials and methods”. 10 μg of human platelet membrane extract (HP) and erythrocyte cell extract (E) was labeled with anti-glycine receptor alpha 1 + alpha 2 antibody. Loading control was done using anti-β-actin antibody

such as aspirin and its analogues, have been used to decrease platelet aggregation (Abrahamsen et al. 1974; Mills et al. 1974); however, many of these drugs have overt side effects which sometimes make compliance difficult. Glycine is a natural, non-toxic amino acid circulating in the 100–200 μM range in blood under normal conditions. Furthermore, dietary glycine has been proven safe in a clinical trial (Rosse et al. 1989). The present investigation showed that administration of glycine in vitro and in vivo inhibited platelet aggregation (Figs. 2, 3) and lengthened bleeding time (Fig. 1). In addition, glycine improves microcirculation (Shechter et al. 1999; Zhong et al. 1996) and inhibits inflammation (Wheeler et al. 1999, 2000; Stachlewitz et al. 2000), thus dietary supplementation with glycine should be of immense benefit in preventing diseases where increased platelet aggregation and thrombosis are involved.

Mechanism by which glycine inhibits platelet aggregation

Different reagents prevent platelet aggregation by various mechanisms. For instance, aspirin inhibits the conversion of arachidonic acid to highly potent platelet aggregating mediators such as thromboxane A₂ (Ferri et al. 1994). PGE₁ and papaverine increase the c-AMP levels in the platelets (Zahavi et al. 1984), and SGB-1543, a phenyl-piperazine derivative, inhibits aggregation by blocking serotonic and adrenergic receptors (Aono and Sakai 1986). Glycine inhibits platelet aggregation most likely by affecting calcium flux. Intracellular calcium plays a central role in regulating platelet function. In the resting state, platelet [Ca²⁺]_i is maintained at low levels (Flores 1996).

When $[Ca^{2+}]_i$ is increased by agonists, either due to Ca^{2+} influx or release from internal stores, platelets become activated and undergo aggregation (Cronberg and Caen 1970; Jancinova et al. 1992). Glycine has been shown to hyperpolarize neurons and Kupffer cells by increasing chloride conductance (Ikejima et al. 1997; Choi et al. 2009; Danober and Pape 1998; Hondo et al. 2011). Glycine binds its receptor (GlyR) which is an anion channel. When this channel is activated, chloride influx increases (Betz and Becker 1988; Blednov et al. 1996; Lynch 2004). Influx of chloride most likely causes hyperpolarization of platelets, and it is known that calcium channels are more difficult to open when the cell is hyperpolarized (Qi et al. 2007). Therefore, glycine may minimize the increase in intracellular calcium, thus inhibiting platelet aggregation. Indeed, the present study showed that platelet aggregation increased after ADP and collagen stimulation, and that this effect was blocked by glycine (Figs. 2, 3). The effect of glycine on platelet aggregation was blocked by strychnine (Fig. 4), a specific antagonist of calcium-gated chloride channel (Young and Snyder 1973; Danober and Pape 1998), indicating that effects of glycine involve this channel. Further, this study demonstrated that, like white blood cells and macrophages (Froh et al. 2002), platelets also contain glycine-gated chloride channels (Figs. 5, 6). Therefore, glycine inhibits platelet aggregation, most likely by activating glycine-gated chloride channels in platelets which decreases the response to a variety of thrombogenic agonists.

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