Cytoprotection by glycine against ATP-depletion-induced injury is mediated by glycine receptor in renal cells[1]

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It is known that glycine protects mammalian cells against ischaemic cell injury by preventing cellular membrane leakage. However, the molecular mechanisms have not yet been clearly elucidated. The purpose of the present study was to clarify whether GlyR (glycine receptor) acts as a key mediator in cytoprotection of glycine. cDNA encoding human GlyRα1 (α1-subunit of glycine receptor) was transfected into HEK-293 cells. The membrane integrity of the cells with or without GlyRα1 was examined by the uptake of marker compounds, the release of LDH (lactate dehydrogenase) and the exclusion of Trypan Blue. Glycine prevented the permeability of 70 kDa dextrans and 140 kDa LDH in the cells in which GlyR was expressed under conditions of ATP depletion. The inhibition of endogenous GlyR expression by RNA interference attenuated the cytoprotection by glycine. Furthermore, the mutation of Tyr^{202} to phenylalanine in GlyRα1 blocked the glycine-mediated cytoprotection, while the mutation of Tyr^{202} to leucine abolished the cytoprotection by strychnine. Our results suggested that the cytoprotection of glycine against ATP-depletion-induced injury might be mediated by GlyR.

Key words: ATP depletion, cytoprotection, gene silence, glycine receptor, mutation, transfection.

INTRODUCTION

ATP depletion causes a series of cell injuries, including plasma-membrane permeabilization, lipid degradation, phospholipase A_2 activation, intracellular calcium elevation, calpain activation, loss of membrane–cytoskeleton linkage and alterations in the function/aggregation of membrane proteins [1–9]. Among these pathological changes, plasma-membrane permeabilization might be a crucial event. When the plasma membrane becomes permeable to macromolecules, injury to the cells becomes irreversible.

It was found that glycine was able to protect proximal tubules from membrane permeabilization during ATP depletion [10]. Glycine increased the chance of cell survival and accelerated the cellular recovery after ischaemia–reperfusion [11,12]. After treatment with glycine, the release of 140 kDa LDH (lactate dehydrogenase) from ATP-depleted renal proximal tubules decreased dramatically. Unlike PI (propidium iodide; molecular mass 668 Da), macromolecular dextrans were unable to penetrate through the glycine-treated membrane under conditions of ATP deprivation [13]. The protective effects of glycine were shown in a broad spectrum of cells, including neurons [12], intestinal epithelium [14], skeletal myocytes [15], renal cells [16] and hepatocytes [17].

The detailed cytoprotection mechanisms of glycine remain to be elucidated. It seems that they are independent of membrane phospholipid metabolism, intracellular calcium changes or calpain activities, mechanisms which have been reported as the major ones involved in the case of most cytoprotectors. Neither an alteration in membrane–cytoskeleton interactions, nor the modification of intracellular pH or the quenching of reactive oxygen species contributed to cytoprotection of glycine [1,3,16]. Glycine is a ligand of GlyR (glycine receptor), which is pentameric anion channel generally consisting of three α and two β isoforms. It is noteworthy that other ligands of GlyR, such as strychnine, share the same cytoprotective effects [16]. Dong et al. [16] found that strychnine prevented cell permeabilization through its interactions with the molecules on the outer surface of the plasma membrane. Hence it is likely that glycine, like strychnine, exerts its cytoprotection via the interaction with GlyR.

The present study was designed to determine whether the cytoprotection of glycine is mediated via GlyR during ATP depletion. GlyRα1 (α1-subunit of glycine receptor) was artificially expressed in HEK-293 cells, which lack endogenous native GlyR. The GlyR-expressing cells were monitored for glycine-induced cytoprotection. Furthermore, the expression of native GlyRα1 in MDCK (Madin–Darby canine kidney) cells was knocked down and the effects on cytoprotection of glycine were evaluated. Finally, the mutation of residue Tyr^{202} in GlyRα1, a key ligand-binding site in GlyR, was carried out in order to test the changes in ligand-induced cytoprotection. Our data demonstrated for the first time that GlyRα1 could be a key mediator in the cytomembrane protection by glycine against ATP-depletion-induced injuries in renal cells.

EXPERIMENTAL

Materials

HEK-293 and MDCK cells were obtained from the A.T.C.C. (American Type Culture Collection, Manassas, VA, U.S.A.). pcDNA3.1(b) vector, DMEM (Dulbecco's modified Eagle's

Abbreviations used: DEPC, diethyl pyrocarbonate; DMEM, Dulbecco's modified Eagle's medium; ECL®, enhanced chemiluminescence; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GlyR, glycine receptor; GlyRα1, α1-subunit of glycine receptor; LDH, lactate dehydrogenase; MDCK, Madin–Darby canine kidney; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; RT, reverse transcription; siRNA, small interfering RNA; TB, Trypan Blue.

[1] This article is dedicated, as a token of our gratitude and to lament his death, to the late Dr Jing Chen, formerly of this University and also of the Department of Life Sciences, College of Arts and Sciences, Indiana State University, Terre Haute, IN, U.S.A.

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medium) and Lipofectamine™ 2000 were obtained from Invitrogen. *Pfu* (*Pyrococcus furiosus*) DNA polymerase and the QuikChange® site-directed mutagenesis kit were from Stratagene. Oligotex Direct mRNA Kit was from Qiagen. BamHI and EcoRI restriction endonucleases, pGEM-T vector and TriPure Isolation Reagent were from Promega. The BCA Protein Assay Kit was from Pierce. Mouse anti-Myc monoclonal antibody was from Santa Cruz. Rabbit anti-GlyRα1 polyclonal antibody was from Chemicon. ECL® (enhanced chemiluminescence) reagent was from Amersham Biosciences. Rotenone, antimycin A, Ionomycin, glucose-free medium, glycine, strychnine, Mouse anti-β-actin monoclonal antibody and luciferase–luciferin ATP assay kit were from Sigma. PI, 3 kDa fluorescein- and 70 kDa Rhodamine B-labelled dextrans were from Molecular Probes. pSilence $1.0U_6$ vector was from Ambion (Europe) Ltd, Huntingdon, Cambridgeshire, U.K. The LDH reagent detection kits were from the Jiancheng Bioengineering Institute, Nanjing, China.

Construction and mutation of vector

The full length of the GlyRα1 gene was amplified by PCR. The vector pCIS (provided by the late Dr. Jing Chen, formerly of the Department of Life Sciences, College of Arts and Sciences, Indiana State University, Haute Terre, IN, U.S.A.), containing GlyRα1 cDNA, was used as a template. PCR primers were designed to contain the BamHI and EcoRI restriction sites, allowing ligation into pcDNA3.1(b) vector. The cDNAs encoding mutants of the Tyr^{202} position in GlyRα1 were constructed by using the QuikChange mutagenesis kit. Two synthetic oligonucleotide primers were complementary to opposite strands of GlyRα1 cDNA containing the mutation in Tyr^{202}. After temperature cycling, the parental DNA template was digested with DpnI. The synthesized DNA containing mutation was selected and then transformed into *Escherichia coli*. Mutations were confirmed by sequencing the cDNA clones.

Cell culture and transfection

HEK-293 and MDCK cells were cultured in DMEM with 10 % (v/v) fetal-calf serum, 100 i.u./ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5 % CO_2. The vectors pcDNA3.1 (b) containing wild-type or mutated GlyRα1 were respectively co-transfected with vector pEGFP-C1 by using Lipofectamine™ 2000. The appropriate ratio between pcDNA3.1(b) and pEGFP-C1 was 1:1. The same vector without the insert was used as control. Briefly, HEK-293 cells were plated on to six-well cultured cell cluster for 24 h. After transfection by addition of 5 μl of Lipofectamine™ 2000, 2 μg of vector including EGFP (enhanced green fluorescent protein) and pcDNA3.1(b) to each well for 5 h, cells were washed with Hanks medium and incubated in 2 ml of normal growth medium. The efficiency of transfection was assayed with flow cytometer and confocal microscope.

Transcription and expression of the GlyRα1 gene

Total RNAs were extracted from cells and purified by using Oligotex Direct mRNA Kits. Two pairs of primers (a1 and a2) were designed to target two sequences of 671–1160 and 455–1216 respectively in the GlyRα1 DNA sequence. They are:

5′-GTTCTTTGCCAACGAGAAGG-3′ (a1 forward)

5′-GAGCACAGTGGTGATGCCTA-3′ (a1 reverse)

5′-CAGGATCAGGCCCAATTTTA-3′ (a2 forward)

5′-ACATAGGACACCTTGGGCAG-3′ (a2 reverse)

PCR reactions were initiated by incubating at 94 °C for 5 min, then 35 cycles of 94 °C/30 s, 57 °C/30 s and 72 °C/1 min. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal standard in the reaction. The RT- (reverse transcription) PCR products were ligated into the pGEM-T vector for DNA sequencing to exclude all possible homologous genes.

The cells were collected into 0.5 ml of lysis buffer consisting of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 % Nonidet P40, 1 mM EDTA, 0.2 mM PMSF and 10 units/ml aprotinin (proteinase inhibitor) at 30 min on ice. Cell lysates were cleared by centrifugation at 12 000 ***g*** for 30 min at 4 °C. Total proteins (30 μg) were subjected to SDS/PAGE and transferred on to a nitrocellulose membrane. The membranes were probed with an mouse anti-myc monoclonal or rabbit anti-GlyRα1 polyclonal antibody at 4 °C overnight with gentle shaking. The immunoreactivity was detected by ECL and analysed by Image-J analysis software.

Search for a human homologous fragment of GlyRα1 in MDCK cells

A total of 14 different sense and antisense primers were designed and paired to human GlyRα1 to different extents to identify the analogue(s) in the MDCK cell line. The RT-PCR products were ligated into the pGEM-T vector for DNA sequencing. Obtained DNA fragment were spliced to get the whole sequence. The sequences of the identified cDNAs were subjected to a BLAST search with the human GlyRα1 gene in the public (NCBI) database.

ATP depletion

The cells were plated at 500 000/well in six-well cell-culture clusters. Mitochondrial oxidative phosphorylation was blocked by the addition of 10 nM rotenone and 100 nM antimycin A. Glycolysis was inhibited when cells were cultured in glucose-free medium containing 100 nM free Ca^{2+} and 5 μM ionomycin. The cellular ATP levels were determined with a luciferase–luciferin ATP assay kit [16].

Measurements of cellular membrane permeability and integrity

The entry into cells of marker compounds (668 Da PI, 3 kDa fluorescein- and 70 kDa Rhodamine-labelled B dextrans) were tested for the cellular membrane permeabilization. The cells were kept under ATP-free conditions for a definite time. The marker compounds were then added to medium for morphological observation under a confocal microscope.

Leakage of intracellular LDH (140 kDa) was measured as an index of plasma-membrane integrity. The extracellular medium was collected before the cells were permeabilized with equal volume of 0.4 % (v/v) Triton. The activities of extracellular and intracellular LDH were measured separately. The extracellular LDH activity was divided by the total LDH activity (extracellular activity plus intracellular activity) to calculate the percentage of LDH release. The TB exclusion assay was performed as described previously [13]. ATP-depleted cells were incubated with 0.2 % (w/v) TB in PBS at 4 °C for 15 min. TB-stained cells were counted under an optical microscope and their percentage of the total cells calculated.

RNA interference with native GlyR

siRNA (small interfering RNA) duplexes were designed to target the identified GlyRα1 DNA sequences in MDCK cells. All potential sequences were analysed and the criteria for application were based on the protocol and searching tool provided by Ambion

(http://jura.wi.mit.edu/pubint/http://iona.wi.mit.edu/siRNAext/). Four targeted regions were selected. The sequences were as follows:

5′-AAGGACTTGAGATACTGCACC-3′ (siRNA1)

5′-AAGGCCGCTTTAACTTCTCTG-3′ (siRNA2)

5′-AACCATGGACTATAGGGTCAA-3′ (siRNA3)

5′-AAGATTGTCCGTAGAGAGGAC-3′ (siRNA4)

The selected siRNA sequences were BLAST-searched from the NCBI database.

The siRNA duplexes were subcloned into pSilence $1.0U_6$ vector and transfected to MDCK cells. The cells were harvested after transfection for 24 or 48 h. The expression of GlyRα1 was detected by RT-PCR and Western blotting.

RESULTS

Expression of GlyR in cells

HEK-293 cell line, which lacked native GlyR [18,19], was used as a cell model in this study. When the vector containing GlyR cDNA was transfected into the cell, the band with 500 bp or 750 bp was detected by RT-PCR with primer a1 or a2. Meanwhile, Western-blot analysis revealed a 48 kDa protein band from transfected HEK-293 cells. This band was the same size as GlyRα1. No similar band was found in wild-type HEK-293 cells (Figure 1).

In the present study, a human homologous fragment of GlyRα1 was identified in MDCK cells. This human homologous fragment differed from human neuronal GlyRα1 in 33 of the 1350 nucleotides and six of the 448 residues as tested by RT-PCR with 14 pairs of the primers. The results of primer a1 and a2 are given in Figure 1(A) and show that both 500 bp and 750 bp bands appeared in an RT-PCR study.

Cytomembrane protection by glycine in ATP-depleted cells

The ATP-depletion cell model was generated by addition of antimycin A (100 nmol/l) and rotenone (10 nmol/l) to glucose-free medium. After 5 min treatment, the level of cellular ATP decreased by more than 95 % in both HEK-293 and MDCK cells (Figure 2). When ATP depletion lasted for 3 h, LDH release in transfected HEK-293 and MDCK cells were 59.18 ± 8.10 % and 80.43 ± 6.53 % separately. After addition of 2 mM glycine to the cell-culture medium, LDH release decreased to 35.15 ± 2.61 % in transfected HEK-293 cells and to 4.96 ± 1.22 % in MDCK cells. No positive change was seen in wild-type HEK-293 cells (Figure 3). The effect of strychnine on LDH release in cell-culture studies was similar to that of glycine. The protection against LDH release from cells appeared to be greater with glycine than with strychnine at the same concentration.

Normally PI and dextrans, the marker compounds of membrane permeability index, are unable to enter the living cells through the plasma membrane. Depletion of ATP caused most cells to be stained by PI, either in the presence or absence of glycine. The entry patterns of dextrans in ATP depletion were similar in all cell types, including HEK-293, transfected HEK-293 and MDCK cells. With 15 min of ATP depletion, few cells were permeable to 3 kDa dextrans. When ATP depletion was prolonged for 30 min, about 5 % of cells were stained with 3 kDa fluorescein-labelled dextrans. After ATP depletion for 60 min, almost 100 % of cells could be stained by 3 kDa dextrans, and 30 % of the cells could be stained by 70 kDa dextrans. Glycine dramatically decreased the entry of 70 kDa dextrans rather than 3 kDa dextrans into MDCK cells and GlyR-transfected cells (Figure 4). This inhibitory effect

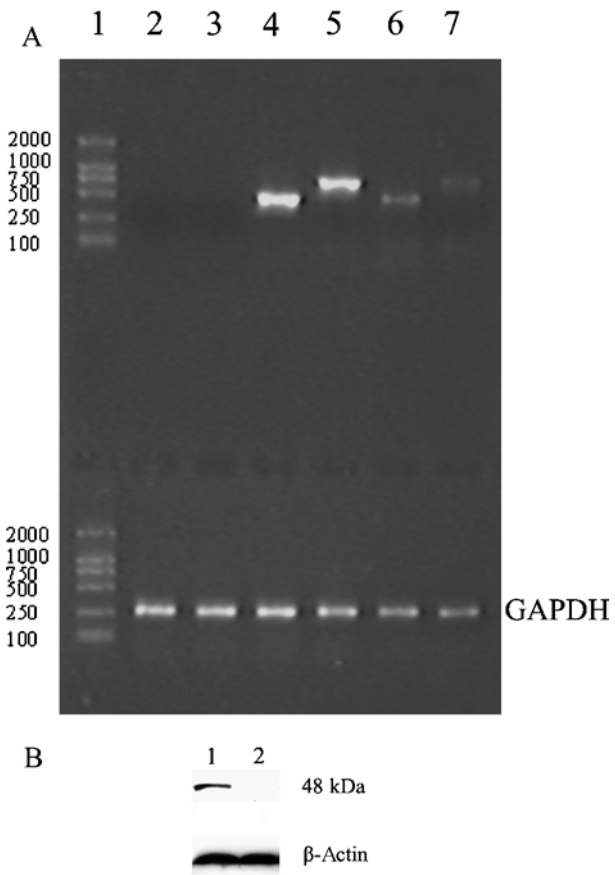

Figure 1 Transcription and translation of GlyRα1 in transfected HEK-293 cells

(**A**) Transcription of GlyRα1 in transfected HEK-293 cells was identified by RT-PCR with primers a1 and a2. The RT-PCR products amplified from MDCK cells were used as a positive control. Lane 1, molecular size markers; lanes 2 and 3, wild-type HEK-293 cells, primer a1 (lane 2) and a2 (lane 3); lanes 4 and 5, transfected HEK-293 cells, primer a1 (lane 4) and a2 (lane 5); lanes 6 and 7, MDCK cells, primer a1 (lane 6) and a2 (lane 7). Values on the extreme left are molecular sizes in bp. (**B**) The expression of GlyRα1 in transfected HEK-293 cells was examined by Western blotting with an anti-Myc antibody. Lane 1, transfected HEK-293 cells; lane 2, wild-type HEK-293 cells.

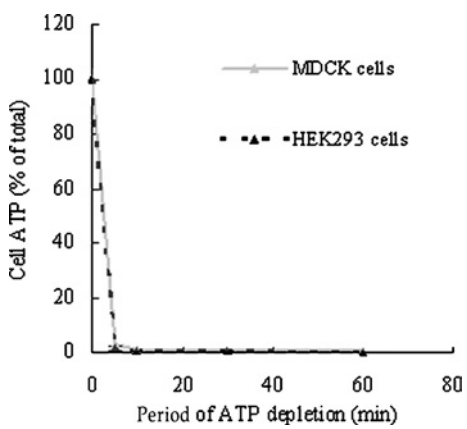

Figure 2 Dynamic 'curves' of ATP depletion in cells

ATP depletion in MDCK or HEK-293 cells was induced by the addition of antimycin A (100 nmol/l) and rotenone (10 nmol/l) to glucose-free medium. The ATP level in cells was determined by luminometry using the luciferin–firefly luciferase reaction.

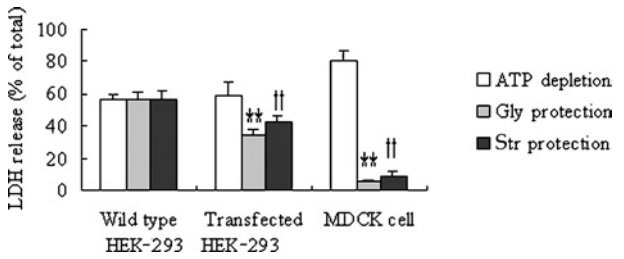

Figure 3 LDH release from ATP-depleted cells

The cells were deprived of ATP for 3 h. LDH release from cells was measured in the presence of 2 mM glycine (Gly) or 2 mM strychnine (Str). On treatment with 2 mM glycine, LDH release decreased from 59.18 ± 8.10 to 35.15 ± 2.61 % in transfected HEK-293 cells and from 80.43 ± 6.53 to 4.96 ± 1.22 % in MDCK cells respectively (mean ± S.D., $n = 6$, $^{**}P < 0.01$ versus LDH release from the cells in the absence of glycine, $\dagger\dagger P < 0.01$ versus LDH release from the cells in the absence of strychnine).

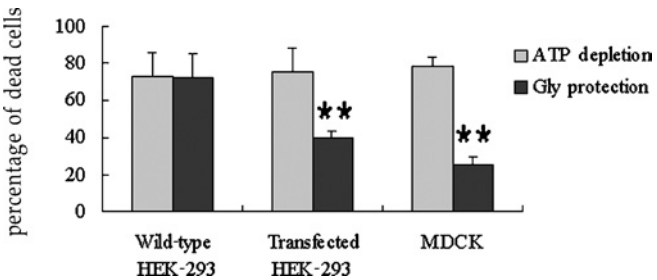

Figure 5 Exclusion of Trypan Blue in cells

Cells were ATP-depleted for 3 h and then incubated with 0.2 % TB in PBS at 4 °C for 15 min. The cytosol stained by TB represents the loss of plasma-membrane integrity and cell death. The positive-staining cells were counted under an optical microscope. Results are means ± S.D. ($n = 6$; $^{**}P < 0.01$ versus ATP-depleted cells in the absence of glycine).

on membrane permeabilization to marker compounds by glycine was not seen in wild-type HEK-293 cells (results not shown).

Consistently the presence of TB in ATP-depleted cytosol was decreased significantly by incubation of glycine with the transfected cells and MDCK cells. The differences were statistically significant ($P < 0.001$). However, the TB staining pattern did not change with the addition of glycine in wild-type HEK-293 cells (Figure 5).

Suppression of cytoprotection by glycine by RNA interference with GlyRα1

When siRNAs were transfected into MDCK cells, GlyRα1 gene transcription was knocked down by various extents. In general, transfection of siRNA into the cell for 24 h showed stronger inhibitory effects on mRNA expression than for 48 h. As to the protein expression in the cell, transfection of siRNA for 48 h showed stronger inhibition (Figure 6A). Most siRNAs resulted in the decrease in target protein by more than 50 %. Among the four siRNAs used, siRNA2 and siRNA3 manifested the highest activities (protein expression in cells decreased by 70 %; Figures 6B and 6C).

To determine whether the interference with GlyRα1 by siRNA could attenuate the protective effects by glycine in MDCK cells, the LDH-release experiments were performed. Under ATP depletion the increase in LDH release paralleled the decrease in GlyRα1 expression. The lower GlyRα1 expression induced by RNA interference supressed the cytomembrane protection by glycine in a statistically signficant manner (Figure 7).

Effect of mutation of GlyRα1 on cytoprotection by glycine

Mutations of Tyr^{202} to phenylalanine or leucine in GlyRα1 in both cases weakened the binding of glycine and strychnine to receptor [20]. To evaluate the potential effects of changes in ligand-binding sites of GlyR on the cytoprotection by glycine, we constructed two mutative plasmids: Tyr^{202} to phenylalanine (named T202F) and Tyr^{202} to leucine (named T202L). Both receptor mutants were expressed in the transfected HEK-293 cells. Under ATP depletion the LDH releases in these cells were 67.33 ± 6.75 % for T202F and 65.33 ± 7.80 % for T202L respectively, which

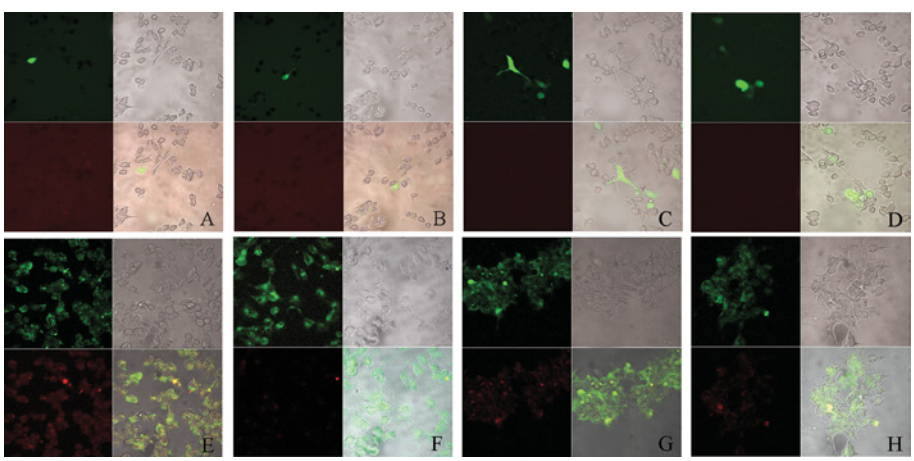

Figure 4 Membrane permeability to the marker compounds under conditions of ATP depletion

The cells were ATP-depleted for 15, 30, or 60 min and then were incubated in a medium containing 0.05 % 3 kDa fluorescein-labelled dextrans (green fluorescence) or 0.05 % 70 kDa tetramethyl-Rhodamine-labelled dextrans (red fluorescence). (**A**)–(**F**) Results for MDCK cells; (**G**)–(**H**) results in GlyRα1-transfected HEK-293 cells. When ATP-depleted for 15 min, cells were hardly peameable to the 3 kDa or 70 kDa dextrans in MDCK cells, no matter whether glycine was present (**B**) or not (**A**, control). ATP depletion for 30 min resulted in 5 % of MDCK cells being stained by 3 kDa fluorescein-labelled dextrans but not by 70 kDa dextrans. Treatment with glycine did not change the staining pattern by 3 kDa dextrans (**C**, control; **D**, treatment with glycine). After ATP depletion for 60 min, all cells were stained by 3 kDa dextran and 30 % of cells were stained by 70 kDa dextrans. The treatment with glycine reduced the positive staining cells by 70 kDa dextrans other than by 3 kDa dextrans (**E**, MDCK cells; **F**, MDCK cells treated with glycine; **G**, transfected HEK-293 cells; **H**, transfected HEK-293 cells treated with glycine).

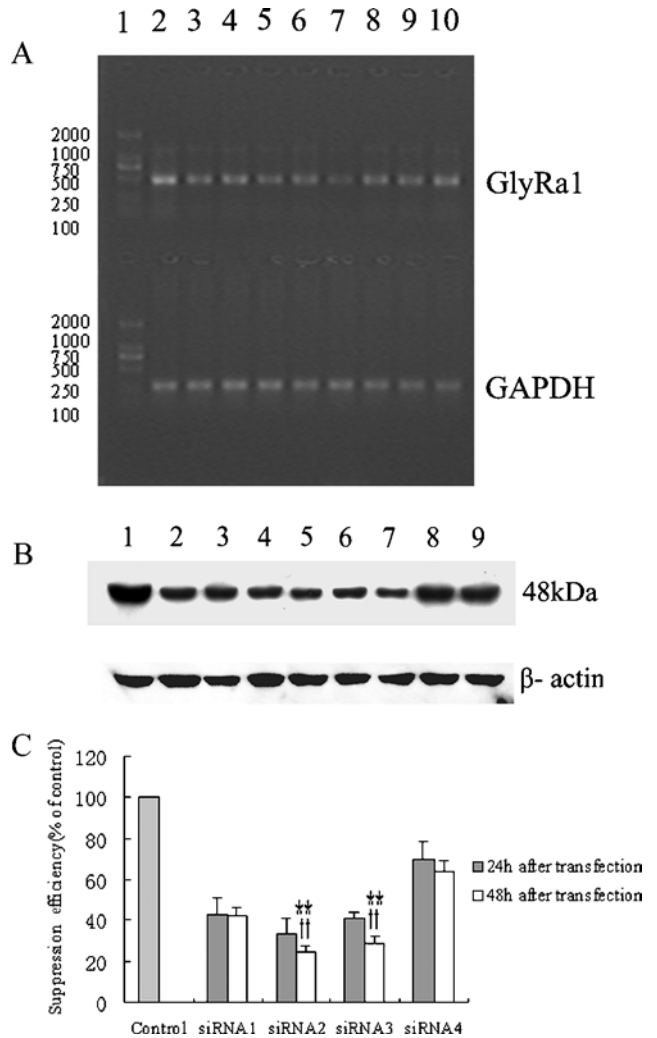

Figure 6 Effects of siRNAs on GlyRα1 transcription and expression in MDCK cells

Four siRNAs corresponding to different regions of GlyRα1 were transfected into MDCK cells for 24 or 48 h. The expression of GlyRα1 was examined by RT-PCR (for mRNA) and Western blotting (for protein). (**A**) Effect of transfection of siRNAs on gene transcription of GlyRα1 in MDCK cells. Lane 1, molecular size markers; lane 2, control; lane 3, siRNA1 (24 h); lane 4, siRNA1 (48 h); lane 5, siRNA2 (24 h); lane 6, siRNA2 (48 h); lane 7, siRNA3 (24 h); lane 8, siRNA3 (48 h); lane 9, siRNA4 (24 h); lane 10, siRNA4 (48 h). Values on the extreme left are molecular sizes in bp. (**B**) Effect of transfection of siRNAs on the protein expression of GlyRα1 in MDCK cells. Lane 1, control; lane 2, siRNA1 (24 h); lane 3, siRNA1 (48 h); lane 4, siRNA2 (24 h); lane 5, siRNA2 (48 h); lane 6, siRNA3 (24 h); lane 7, siRNA3 (48 h); lane 8, siRNA4 (24 h); lane 9, siRNA4 (48 h). (**C**) The inhibitory effects of siRNAs on GlyRα1 expression in MDCK cells. The results were analysed by Image-J software. The GlyRα1 expression in control cells was defined as 100%. Results are means ± S.D. ($n = 3$; $^{**}P < 0.01$ versus GlyRα1 expression in siRNA4-transfected cells; $\dagger\dagger P < 0.01$ versus GlyRα1 expression in the cells transfected with siRNA for 24 h.)

were the same as for the cells expressing the wild-type receptors. Glycine prevented LDH release from T202L-expressed cells, but showed little effect on T202F-expressing cells. By contrast, strychnine showed inhibition of LDH release in T202F-expressing cells, but only a slight effect on that of T202L-expressing cells (Figure 8).

DISCUSSION

As a ligand of GlyR, glycine is known to be a cytoprotector against ischaemia-induced membrane permeabilization. This cytoprotection is postulated to be mediated by GlyR, because other ligands of GlyR, such as strychnine and β-alanine, give a cytomembrane protection similar to that given by glycine [16,21–22]. However, this hypothesis is challenged by a few facts. For example, glycine is also a co-agonist of glutamate to NMDA (*N*-methyl-D-aspartate) receptor [23]. The competitive antagonists for the glycine-binding site in the NMDA receptor show neuroprotection to different extents [24,25]. It implies that the NMDA receptor may be engaged in glycine-induced cytoprotection. The tests on cultured hepatic sinusoidal endothelial cells indicated that ATP depletion induced a loss of plasma-membrane integrity by opening a glycine-sensitive channel, the 'death channel'. Glycine showed its ability to cytoprotect via blockage of the death channel without interacting with GlyR [26]. Furthermore, the alteration of Na^+ homoeostasis resulted in irreversible hepatocyte injury during ATP depletion [27]. By means of inhibition of Na^+ influx independent of the activation of Cl^- channels, glycine performed its cytoprotective function [17]. Thus a clear explanation of the mechanisms behind cytoprotection by glycine awaits further verification. The goal of the present study was to determine whether GlyR mediated cytoprotection by glycine. Consisting of three α1 and two β isoforms, adult GlyR is classified as a member of the ligand-gated ion-channel family [28]. The formation of a Cl^- channel basically relies on the α1 subunit. The recombinant homomeric GlyRα1 receptor has been widely used as a model

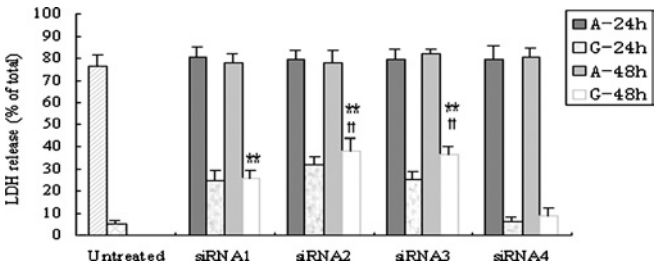

Figure 7 Effects of RNA interference with GlyRα1 on LDH release from MDCK cells

After transfection with siRNAs for 24 or 48 h, MDCK cells were ATP-depleted. Compared with control cells, LDH release from the cells increased significantly in the presence of glycine. Results are means ± S.D. ($n = 6$; $^{**}P < 0.01$ versus LDH release from the untransfection cells in the presence of glycine). Transfection with siRNA into cells for 48 h caused stronger inhibition of the protection by glycine against LDH release from cells than did that for 24 h. siRNA2 and siRNA3 showed stronger inhibition of the cytoprotection by glycine. ($\dagger\dagger P < 0.01$ versus the LDH release from the cells transfected for 24 h). In the boxed key, A refers to ATP depletion and G refers to glycine depletion.

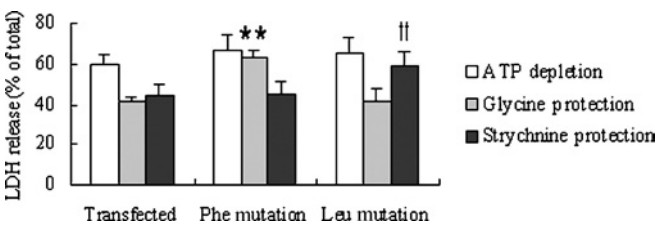

Figure 8 Effect of GlyRα1 mutation on LDH release in transfected cells

HEK-293 cells were transfected with GlyRα1 or the Tyr^{202} receptor mutants. LDH release from transfected cells was assayed under conditions of ATP depletion for 3 h in the absence or presence of 2 mmol/l glycine or strychnine. Replacement of the residue Tyr^{202} by phenylalanine (Phe mutation) in GlyRα1 profoundly decreased protection by glycine, but only slightly affected that of strychnine. Replacement of Tyr^{202} by leucine (Leu mutation) inhibited protection by strychnine, but not by glycine. Results are means ± S.D. ($n = 6$; $^{**}P < 0.01$ versus the cells transfected with GlyRα1 in the presence of glycine; $\dagger\dagger P < 0.01$ versus the cells transfected with GlyRα1 in the presence of strychnine).

of GlyR in *in vitro* studies [19,29–30]. The β subunit controlled the stable $\alpha1/\beta$ stoichiometry and interacted with gephyrin to cluster and anchor the receptor in the plasma membrane [31]. The recombinant homomeric GlyRβ alone did not function as a Cl^- channel. In the present study we constructed genetically engineered Glyα1-expressing HEK-293 cells in order to identify the roles of GlyR in glycine-induced cytoprotection. Our findings demonstrated that GlyR contributed to the cytoprotection by glycine. First, cytoprotection by glycine took place in MDCK cells in which GlyR was expressed endogenously. No similar effects were found in the wild-type HEK-293 cells in which GlyR was lacking. When GlyRα1 was artificially expressed in HEK-293 cells, cytoprotection by glycine occurred. Secondly, the interference with endogenous GlyRα1 expression in MDCK cells by RNA interference attenuated the protective effects of glycine against ATP depletion. The more GlyRα1 expression was knocked down, the weaker was the cytoprotection afforded by glycine. Thirdly, the destruction of the ligand-binding site of GlyRα1 abolished the binding of glycine to GlyR in transfected cells, while, at the same time, the cytoprotection of glycine was impaired dramatically. Thus this evidence strongly supported the notion that GlyR plays an important role in the cytoprotection by glycine against ATP depletion.

The mechanisms underlying the GlyR-dependent cytoprotection are not yet clear. As a ligand-gated Cl^- channel, the activation of GlyR depends on binding to glycine. It initiates a rapid allosteric transition within the transmembrane domain and results in a Cl^- influx that can be completely shut off by strychnine, an antagonist of GlyR [32,33]. However, strychnine also displays a cytoprotective function which is similar to that displayed by glycine. The fact that both the agonist and the antagonist of Cl^- channel are cytoprotective implies that the Cl^- channel might be irrelevant to the cytoprotective effect mediated by GlyR.

It is known that the residue Tyr^{202} in GlyRα1 is a critical site for binding with the ligands [20]. We found that when Tyr^{202} was replaced by phenylalanine in GlyR, both the binding of the receptor to glycine and glycine-induced cytoprotection was profoundly decreased. The mutation of Tyr^{202} to leucine in GlyR specifically abolished the binding of strychnine to the receptor, but cytoprotection by glycine was unaffected. Consistent with this, the cytoprotection by strychnine was also shown to rely only on the binding to GlyR. Thus it is possible that the ligands of GlyR might exert their cytoprotection by directly binding to the receptor independently of Cl^- channel activation.

How does the interaction between GlyR and its ligands cause the changes in cell membrane characterized by the cytoprotection against ATP depletion? The focus had been on changes in ion channels, especially those in the glycine-sensitive anion channel that is independent of Cl^- channel [26]. The opening of the glycine-sensitive anion channel caused cell swelling, bleb formation and stretching of the plasma-membrane bilayer, which subsequently led to porous defects in the plasma membrane [9,26,34]. However, the cation channel, especially the Na^+ channel also needs to be considered. In the early phase of ATP depletion, Na^+ and K^+ gradients collapse, owing to inhibition of the Na^+,K^+-ATPase and the opening of univalent-cation channels. Although Na^+ influx did not seem to cause membrane disruption directly, it did induce the changes in osmotic pressure and cell swelling [35]. By preventing the formation of non-specific leaks for small ions, including Na^+, glycine acted as a cytoprotector [17].

It was postulated that a rapid disruption of the cell membrane occurred when the glycine-sensitive death channel was opened [26]. In the present study we found that the change in membrane permeability progressed. The membrane permeability to smaller molecules was more sensitive to ATP depletion in cells. The object of cytoprotection by glycine might be the leakage of macromolecules greater in size than the than 3 kDa dextrans. This effect was similar to that of homobifunctional NHS (N-hydroxysuccinimide)-ester cross-linkers, which prevented the ATP-depleted renal-proximal- tubule membrane permeability to 70 kDa dextrans instead of 3 kDa dextrans [13]. Therefore, mediation by GlyR in the cytoprotection by glycine might be involved in the rearrangement of plasma-membrane proteins to form water-filled pores for the leakage of certain sizes of macromolecules [9,26,34]. Upon binding with the ligands, GlyR would undergo a rapid allosteric transition via a series of concerted molecular motions that would delay the breakage of membrane linkages as well as the formation of the large 'pores' or 'channels'.

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