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BRIEF ARTICLE

Effects of glycine on phagocytosis and secretion by Kupffer cells *in vitro*

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Abstract

AIM: To investigate the effects and mechanisms of action of glycine on phagocytosis and tumor necrosis factor (TNF)-α secretion by Kupffer cells *in vitro*.

METHODS: Kupffer cells were isolated from normal rats by collagenase digestion and Percoll density gradient differential centrifugation. After culture for 24 h, Kupffer cells were incubated in fresh Dulbecco's Modification of Eagle's Medium containing glycine (G1: 1 mmol/L, G2: 10 mmol/L, G3: 100 mmol/L and G4: 300 mmol/L) for 3 h, then used to measure phagocytosis by a bead test, TNF-α secretion after lipopolysaccharide stimulation by radioactive immunoassay, and microfilament and microtubule expression by staining with phalloidin-fluorescein isothiocyanate (FITC) or a monoclonal anti-α tubulin-FITC antibody, respectively, and evaluated under a ultraviolet fluorescence microscope.

RESULTS: Glycine decreased the phagocytosis of Kupffer cells at both 30 min and 60 min ($P < 0.01$, $P < 0.05$). The numbers of beads phagocytosed by Kupffer cells in 30 min were 16.9 ± 4.0 (control), 9.6 ± 4.1 (G1), 12.1 ± 5.7 (G2), 8.1 ± 3.2 (G3) and 7.5 ± 2.0 (G4), and were 22.5 ± 7.9 (control), 20.1 ± 5.8 (G1), 19.3 ± 4.8 (G2), 13.5 ± 4.7 (G3) and 9.2 ± 3.1 (G4) after 60 min. TNF-α secretion by Kupffer cells in G1 (0.19 ± 0.03), G2 (0.16 ± 0.04), G3 (0.14 ± 0.03) and G4 (0.13 ± 0.05) was significantly less than that in controls (0.26 ± 0.03, $P < 0.01$), and the decrease in secretion was dose-dependent ($P < 0.05$). Microfilaments of Kupffer cells in G2, G3 and G4 groups were arranged in a disorderly manner. The fluorescence densities of microtubules in G1 (53.4 ± 10.5), G2 (54.1 ± 14.6), G3 (64.9 ± 12.1) and G4 (52.1 ± 14.2) were all lower than those in the controls (102.2 ± 23.7, $P < 0.01$), but the decrease in microtubule fluorescence density was not dose-dependant.

CONCLUSION: Glycine can decrease the phagocytosis and secretion by Kupffer cells *in vitro*, which may be related to the changes in the expression of microfilaments and microtubules induced by Kupffer cells.

Key words: Glycine; Kupffer cell; Phagocytosis; Secretion

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INTRODUCTION

Glycine has been well characterized in the spinal cord as an inhibitory neurotransmitter which activates expression of the glycine-gated chloride channel (GlyR) in postsyn-

aptic membranes. Kupffer cells contain a GlyR similar to that described previously in the central nervous system[1,2]. Many studies have shown that dietary or intravenous glycine has a protective effect in rat models against endotoxic shock, hemorrhagic shock, liver ischemia-reperfusion, liver transplantation, and alcohol-induced liver injury and is most likely to exert this effect by inactivating the Kupffer cells *via* this newly identified GlyR[3-10]. Our previous studies also indicated that glycine protected rats from thioacetamide-induced liver injury and intestinal endotoxemia[11,12]. The mechanism involved may be related to inhibition of the release of pro-inflammatory cytokines by Kupffer cells induced by glycine. *In vivo* and *in vitro* experiments have found that glycine inhibits the secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in Kupffer cells[13-15]. However, the impact of glycine on phagocytosis by Kupffer cells has not been reported, and the mechanisms underlying the effect of glycine on TNF-α secretion by Kupffer cells have not been fully understood. Our *in vitro* study showed that lipopolysaccharide (LPS) probably enhanced or inhibited the phagocytosis of Kupffer cells by acting through mechanisms involving microfilaments or microtubules[16]. This study aimed to investigate the effects of glycine on phagocytosis and the mechanisms underlying TNF-α secretion by Kupffer cells *in vitro*.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 300-330 g were obtained from the Experimental Animal Center of Shanxi Medical University (China). All animals were fed with standard laboratory chow and water was available *ad libitum*. The experimental protocols were approved by the Shanxi Animal Research Ethics Committee.

Reagents

Polystyrene beads (1.1 μm), monoclonal anti-α tubulin-fluorescein isothiocyanate (FITC) conjugate, LPS (*Escherichia coli* Serotype 0128:B12), collagenase Ⅳ, phalloidin-FITC, hydroxyethyl piperazine ethanesulfonic acid (HEPES) Percoll, and Dulbecco's Modification of Eagle's Medium (DMEM) were purchased from Sigma (St. Louis, United States); a radioimmunoassay kit for TNF-α measurement was purchased from the Radio-Immunity Institute of the Chinese Liberation Army Omni-hospital (Beijing, China); glycine, sodium pentobarbital, fetal bovine serum (FBS), penicillin G, streptaquaine, insulin, glutamine, trypan blue, and all other reagents not specifically mentioned elsewhere were prepared by Beijing Chemical Inc. (Beijing, China).

Isolation and culture of Kupffer cells

Kupffer cells from Wistar rats were isolated by collagenase digestion and differential centrifugation, using Percoll density gradients as described previously with slight modifications[17]. Briefly, the liver was perfused *in situ* through the portal vein with Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS) containing 0.5 mmol/L ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N- tetraacetic acid (EGTA) at 37 ℃ for 5 min at a flow rate of 26 mL/min. Subsequently, perfusion was performed with HBSS containing 0.05% collagenase Ⅳ at 37 ℃ for 5 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze, and the filtrate was centrifuged twice at 50 × *g* at 4 ℃ for 3 min to remove parenchymal cells. The nonparenchymal cell fraction was washed with buffer and centrifuged on a density cushion of Percoll at 1000 × *g* at 4 ℃ for 20 min to obtain the Kupffer cell fraction, and the cells obtained were washed with buffer again. The viability of isolated Kupffer cells was determined by trypan blue exclusion and routinely exceeded 90%. Cells were seeded onto 24-well culture plates (Corning, NY) or 25 mm × 25 mm glass coverslips at a density of 1×10^6 or 5×10^5 and cultured in DMEM supplemented with 10% FBS, antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin sulfate), 0.1 U/100 mL insulin and 15 mmol/L glutamine at 37 ℃ with 5% CO_2. Non-adherent cells were removed after 1 h by replacing the culture medium. All adherent cells phagocytosed latex beads and stained positive for catalase, confirming that they were Kupffer cells, and cells were cultured for 24 h before experiment.

Effects of glycine on Kupffer cells

Cells were seeded onto 24-well plates and 12 mm × 12 mm glass coverslips, and incubated with fresh medium containing glycine (G1: 1 mmol/L, G2: 10 mmol/L, G3: 100 mmol/L and G4: 300 mmol/L) at 37 ℃ with 5% CO_2 for 3 h. Phagocytosis and expression of microfilaments and microtubules by Kupffer cells were measured by the bead phagocytosis test, fluorescence staining and immunofluorescence staining, as described below.

Measurement of phagocytosis by Kupffer cells

Phagocytosis by Kupffer cells was evaluated by the Kupffer cell's ability to ingest polystyrene beads according to the modified method of Hirose *et al*[18]. Briefly, cells were seeded onto 12 mm × 12 mm glass coverslips or glass plates and incubated with fresh medium containing 0.05% polystyrene beads for 30 min or 60 min at 37 ℃ with 5% CO_2. Following vigorous pipetting to remove non-phagocytosed latex beads, the coverslips or glass plates were washed 3 times with PBS and fixed with 2% formaldehyde or methanol for 5 min. After staining by Giemsa's method for 15 min at room temperature and washing 3 times with PBS, the coverslips were inverted onto glass slides and observed under phase contrast microscope. The mean number of latex beads phagocytosed by each Kupffer cell was counted in at least 20 Kupffer cells per field at magnification of 200 times, 5 fields per coverslip in 6 coverslips.

Measurement of Kupffer cell secretion

Kupffer cells were seeded into 24-well plates at a density of 1×10^6/well and incubated with fresh DMEM

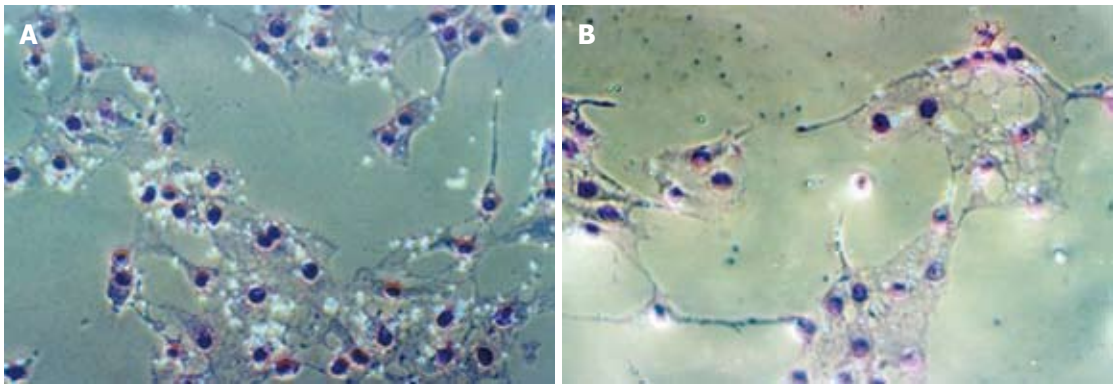

Figure 1 Effects of glycine on phagocytosis by Kupffer cells *in vitro*. A: Phagotosis by Kupffer cells in the control group 30 min after the addition of latex beads, 200×; B: Phagotosis by Kupffer cells in group G3 30 min after the addition of latex beads, 200×.

containing 100 ng/mL LPS for 60 min at 37 ℃ with 5% CO_2. At the end of this period, the medium was collected, centrifuged at 1000 × *g* at 4 ℃ for 10 min, and the supernatant was stored at -80 ℃ until used for TNF-α assay. TNF-α in medium was measured using the radioimmunoassay kit. The levels of TNF-α in the wells represented the secretion of Kupffer cells.

Measurement of microfilament expression by Kupffer cells

Kupffer cells were stained with phalloidin-FITC according to the modified method of Wulf *et al*[19]. Briefly, Kupffer cells were seeded onto 12 mm × 12 mm glass coverslips at a density of 5×10^5 (1×10^4 to 2×10^4 cells/coverslip), fixed with 2% formaldehyde for 20 min and extracted with 0.5% Triton X-100 for 15 min. The fixed cells were then washed 3 times with PBS (10 mmol/L, pH 7.4) and stained with phalloidin-FITC for 45 min at room temperature in the dark. They were then washed for a further 3 times with PBS, the coverslips were inverted onto mounting medium applied to glass slides, and they were observed and photographed under a ultraviolet (UV) fluorescence microscope with a high magnification of 400 times. Mounted preparations could be stored in the dark at 2 ℃-8 ℃.

Measurement of microtubules in Kupffer cells

Microtubules in Kupffer cells were stained with a monoclonal anti-α tubulin-FITC antibody according to the method recommended by the producer. Briefly, Kupffer cells were seeded onto 12 mm × 12 mm glass coverslips at a density of 5×10^5 (1×10^4 to 2×10^4 cells/coverslip). They were then fixed with cold methanol for 10 min at -20 ℃ and rinsed twice with cold acetone (-20 ℃) for 10 s, then the cell layer was rehydrated in PBS (10 mmol/L, pH 7.4) for at least 30 min and stained with monoclonal anti-α tubulin-FITC (1:25 diluted with PBS containing 1% bovine serum albumin) in a dark-room for 60 min at room temperature. The stained cells were washed 3 times with PBS, the coverslips were inverted onto mounting medium applied to glass slides and observed and photographed under a UV fluorescence microscope. Mounted preparations could be stored in the dark at 2-8 ℃. The fluorescence density was measured in 10 cells using the MIAS-300 picture analysis system from at least 5 fields in each picture at a high magnification of 400 times.

Table 1 Effects of glycine on phagocytosis by Kupffer cells *in vitro* (mean ± SD)

Groups	Beads observed in Kupffer cells (n = 6)	
	30 min	60 min
Control	16.9 ± 4.0	22.5 ± 7.9
G1	9.6 ± 4.1[b]	20.1 ± 5.8
G2	12.1 ± 5.7[a]	19.3 ± 4.8
G3	8.1 ± 3.2[b]	13.5 ± 4.7[b,c]
G4	7.5 ± 2.0[b]	9.2 ± 3.1[b,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control; [c]$P < 0.05$, [d]$P < 0.01$ *vs* G1.

Statistical analysis

All results were expressed as mean ± SD. Statistical differences between means were analyzed by one-way analysis of variance or *t* test using the SPSS 12.0 statistical package. Statistical significance level was set at $P < 0.05$.

RESULTS

Effects of glycine on phagocytosis by Kupffer cells

When incubated in 5% CO_2 with fresh medium containing glycine at 37 ℃ for 30 min or 60 min, phagocytosis by Kupffer cells decreased significantly. The number of beads phagocytosed by Kupffer cells in groups G3 and G4 was less than that of group G1 in 60 min. There were no significant differences in the amount of beads phagocytosed by Kupffer cells among the G2, G3 and G4 groups (Table 1 and Figure 1).

Effects of glycine on TNF-α secretion by Kupffer cells

When incubated in 5% CO_2 with fresh medium containing glycine at 37 ℃ for 3 h, TNF-α secretion by Kupffer cells decreased significantly, and the decrease in secretion was dose dependent. TNF-α concentrations detected in the medium of groups G3 and G4 were significantly lower than in the medium of group G1 (Table 2).

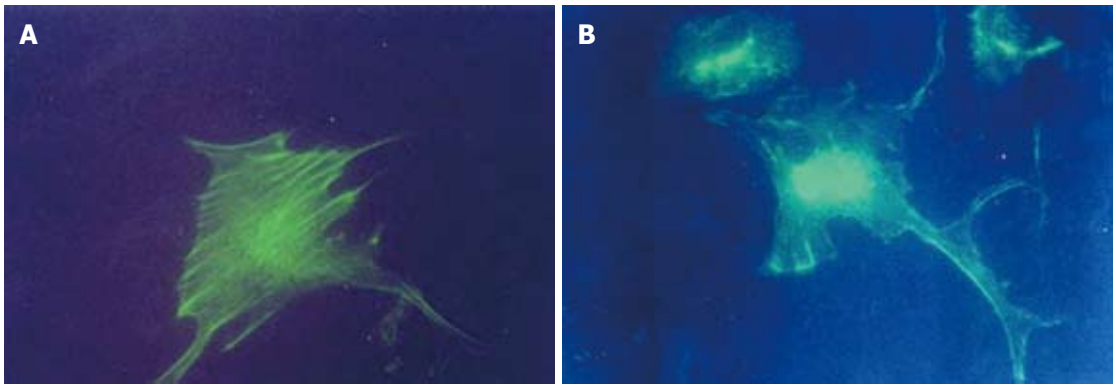

Figure 2 Effects of glycine on expression of microfilaments by Kupffer cells *in vitro*. A: The expression of microfilaments by Kupffer cells in the control group, stained with Phalloidin-fluorescein isothiocyanate (FITC), 400×; B: The expression of microfilaments by Kupffer cells in group G3, stained with Phalloidin-FITC, 400×.

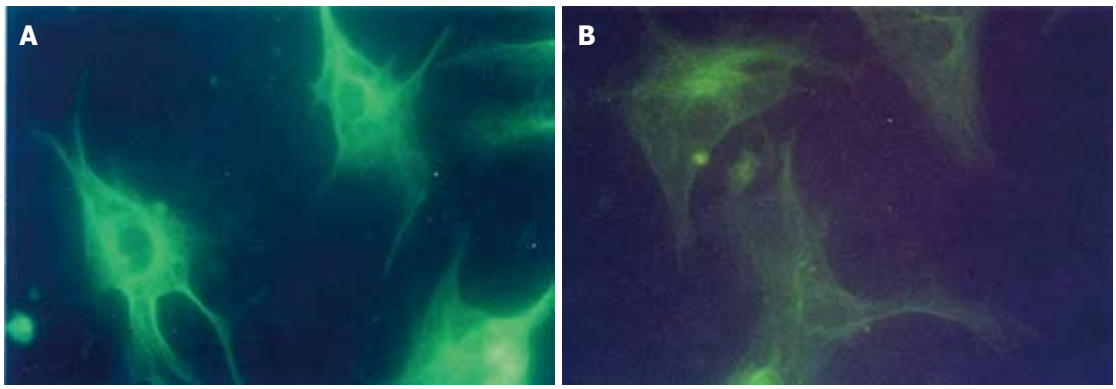

Figure 3 Effects of glycine on expression of microtubules by Kupffer cells *in vitro*. A: The expression of microtubules by Kupffer cells in the control group, stained with monoclonal anti-α tubulin-fluorescein isothiocyanate (FITC) conjugate, 400×; B: The expression of microtubules by Kupffer cells in group G3, stained with monoclonal anti-α tubulin-FITC conjugate, 400×.

Table 2 Effects of glycine on tumor necrosis factor-α secretion and microtubule density of Kupffer cells *in vitro* (n = 6) (mean ± SD)

Groups	TNF-α detected in medium (μg/mL)	Microtubule density of Kupffer cells
Control	0.26 ± 0.03	102.2 ± 23.7
G1	0.19 ± 0.03[d]	53.4 ± 10.5[d]
G2	0.16 ± 0.04[d]	54.1 ± 14.6[d]
G3	0.14 ± 0.03[a,d]	64.9 ± 12.1[d]
G4	0.13 ± 0.05[a,d]	52.1 ± 11.4[d]

[a]P < 0.05 *vs* G1; [d]P < 0.01 *vs* control. TNF: Tumor necrosis factor.

Effects of glycine on microfilaments of Kupffer cells

After 3 h incubation in 5% CO_2 with fresh medium containing glycine at 37 ℃, Kupffer cells stained with FITC-Phalloidin did not demonstrate organized microfilaments in groups G2, G3 or G4. There were no significant differences in the microfilament fluorescence densities among Kupffer cells in control, G1, G2, G3 and G4 groups (Figure 2).

Effects of glycine on microtubules of Kupffer cells

Following 3 h incubation in fresh medium containing glycine at 37 ℃ with 5% CO_2, Kupffer cells were stained with monoclonal anti-α tubulin-FITC. A significant decrease in the fluorescence density of microtubules was observed in Kupffer cells incubated with glycine as compared with the controls. However, the fluorescence density of the microtubules did not show a dose-dependent decrease among G1, G2, G3 and G4 groups (Table 2 and Figure 3).

DISCUSSION

Kupffer cells are the main component of the host monocyte-macrophage system, and their two main functions are phagocytosis and secretion. There is much evidence indicating that activation of Kupffer cells and their production of pro-inflammatory cytokines contribute to the pathogenesis of different liver injuries, including alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) and liver failure among others[20-22]. Tsujimoto *et al*[23] showed that phagocytic activity of Kupffer cells was decreased in a rat model of nonalcoholic steatohepatitis. Glycine is a non-essential amino acid and an inhibitory neurotransmitter in the central nervous system. Many studies have shown that dietary or intravenous glycine can protect against a variety of liver injuries[3-10]. In this study, we found that glycine decreases the phagocytosis and secretion of Kupffer cells *in vitro*.

Effects of glycine on phagocytosis by Kupffer cells

The mechanisms of Kupffer cell phagocytosis are still not completely understood. The ruffling of the cell membrane and formation of pseudopodia may play an important role and these effects are believed to be accomplished by the cytoskeleton. In the cytoskeleton, actin-myosin interaction through the calcium-calmodulin system plays a major role in this activity[24]. In this system, intracellular Ca^{2+} combines with calmodulin to form the active calcium-calmodulin complex, which activates an enzyme, myosin light chain kinase, to phosphorylate the light chain of myosin. Phosphorylated myosin, but not unphosphorylated myosin, can interact with actin to induce activity of the cell membrane and pseudopodia, leading to phagocytosis. This process is reversible, in that a phosphatase can catalyze dephosphorylation of myosin, restoring it to a form that can not be activated by actin.

Previous studies have shown that integrity of the cytoskeletal system is important for phagocytosis of Kupffer cells. Depolymerization of the cytoskeleton decreased phagocytosis by Kupffer cells[25-27]. However, the effects of glycine on phagocytosis by Kupffer cells have not been reported.

The present experiments show that glycine decreases phagocytosis by Kupffer cells *in vitro*, causes disordering of the microfilaments in Kupffer cells, and reduces their expression of microtubules. All these results show that glycine can decrease the phagocytosis of Kupffer cells by acting on the microfilaments and microtubules.

Effects of glycine on secretion by Kupffer cells

Some studies have shown that both CD_{14} and non-CD_{14} mechanisms are involved in the TNF-α secretion of monocytes and Kupffer cells, and that both endocytosis and Ca^{2+} are required for endotoxin-stimulated TNF-α release by Kupffer cells in rats[28-30]. Previous studies have shown that glycine can protect against many injuries and illnesses in rat models, most likely by inactivating Kupffer cells and decreasing TNF-α secretion[3-15]. An *in vitro* study has shown that glycine prevents the increases in $[Ca^{2+}]_i$ caused to LPS by activating chloride influx-reduced synthesis and release of toxic mediators by Kupffer cells[2]. Thus, glycine can activate the chloride influx, prevent the increases in $[Ca^{2+}]_i$ and reduce the TNF-α secretion of Kupffer cells.

Other studies have demonstrated the involvement of a microtubule-dependent mechanism in TNF-α secretion by monocytes. Taxol, a microtubule-stabilizing antineoplastic agent, induced expression of tumor TNF-α in macrophages[31]. Microtubule-disrupting agents such as colchicine had opposite effects on TNF-α production[32-34]. The present experiments showed that glycine significantly decreased TNF-α secretion and microtubule expression. Some of our results are consistent with previous reports[13-15], leading us to believe that glycine can prevent TNF-α secretion by Kupffer cells through disruption of microtubules.

In summary, glycine decreases both phagocytosis and secretion by Kupffer cells *in vitro*, which is probably related to glycine-induced changes in expression of microfilaments and microtubules in Kupffer cells.

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COMMENTS

Background

Activated Kupffer cells are most likely involved in the pathogenesis of different liver injuries. Glycine generally is considered as a protective agent for liver injuries. The mechanism may be related to the fact that glycine inhibits the release of pro-inflammatory cytokines by Kupffer cells. So, it is very important to clarify the impact of glycine on the phagocytosis and secretion by Kupffer cells.

Research frontiers

It is believed that cytoskeleton plays a vital physiological role in phagocytosis by Kupffer cells, and depolymerization of cytoskeleton decreases the phagocytosis by Kupffer cells. Glycine protects against liver injuries by preventing the elevation of intracellular Ca^{2+} and reducing pro-inflammatory cytokines production by Kupffer cells. But the impact of glycine on phagocytosis by Kupffer cells is still unclear, and the mechanisms of glycine on tumor necrosis factor-α secretion by Kupffer cells have not been completely understood.

Innovations and breakthroughs

This is the first study to report that glycine decreases the phagocytosis of Kupffer cells by acting on the microfilaments and microtubules *in vitro*.

Applications

This study suggests that glycine may be an effective agent which could provide a future strategy for therapeutic intervention in the treatment of liver injuries induced by activated Kupffer cells.

Peer review

It is an interesting study with appropriate methodology and the results are clear and of great importance.

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