MICROCIRCULATION AND OXYGEN METABOLISM IN LIVER PATHOPHYSIOLOGY

Glycine as a potent anti-angiogenic nutrient for tumor growth

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Abstract

Accumulating lines of evidence suggest a possibility that glycine is useful as an immuno-modulating amino acid. Glycine most likely prevents the lipopolysaccharide (LPS)-induced elevation of intracellular Ca2+ concentration in Kupffer cells, thereby minimizing LPS receptor signaling and cytokine production. Moreover, it was reported that dietary glycine inhibits the growth of tumors. Vascular endothelial growth factor (VEGF) plays a critical role in cancer progression by promoting new blood vessel formation. Activation of VEGF receptor has been shown to result in activation of phospholipase C-γ and increases in intracellular Ca2+ concentration. The VEGF-induced cell proliferation is dependent on intracellular Ca2+ concentration. The effects of glycine on VEGF-induced increases in intracellular Ca2+ concentration in endothelial cell line (CPA) were studied. The VEGF increased intracellular Ca2+ concentration rapidly, but glycine blunted increases in intracellular Ca2+ concentration due to VEGF. Further, the inhibitory effects of glycine were prevented by low concentrations of strychnine (1 μmol/L) or incubation with chloride-free buffer. Moreover, glycine increased influx of radiolabeled chloride into CPA cells approximately 10-fold. Furthermore, mRNA 92% identical to the β-subunit of the glycine-gated chloride channel from spinal cord was identified in endothelial cells using reverse transcription–polymerase chain reaction. Finally, glycine significantly diminished serum-stimulated proliferation and migration of endothelial cells. These data indicate that the inhibitory effect of glycine on growth and migration of endothelial cells is due to activation of a glycine-gated chloride channel. This hyperpolarizes the cell membrane and blocks influx of Ca2+, thereby minimizing growth factor-mediated signaling. Therefore, glycine can be used not only for treatment of inflammation, but also for chemoprevention and treatment of carcinoma.

Key words

angiogenesis, calcium signal, endothelial cell, vascular endothelial growth factor.

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Introduction

Angiogenesis plays a major role in the progressive growth of solid tumors that are dependent upon the supply of oxygen and nutrients from new blood vessels. Indeed, inhibition of angiogenesis is an important target for tumor treatment. Angiogenesis is a multistage process that involves release of angiogenic factors from several cell types, including tumor cells, proliferation and migration of endothelial cells and synthesis of vascular basement membrane. The vascular endothelial growth factor (VEGF) family of cytokines and their receptors play a central role in these processes. It is secreted by various tumor cells, is expressed by several normal cells during development, and has a highly specific mitogenic effect on endothelial cells. Activation of the VEGF receptor has been shown to result in tyrosine phosphorylation, activation of phospholipase Cy and increases in inositol 1,4,5-trisphosphate and intracellular calcium.

Glycine, a simple non-essential amino acid, is an inhibitory neurotransmitter in the central nervous system that acts on a glycine-gated chloride channel. It was reported that a similar chloride channel was discovered in Kupffer cells, alveolar macrophages and neutrophils. Glycine inhibits endotoxin-induced increases in intracellular calcium and tumor necrosis factor-α released by Kupffer cells. The mechanism of this effect involves hyperpolarization of the cell membrane by increased chloride influx. This makes voltage-dependent Ca2+ channels more difficult to open and prevents increases in intracellular Ca2+ from a variety of agents.

Dietary glycine blunted both the growth of B16 melanoma tumors in mice and development of liver tumors caused by the non-genotoxic carcinogen and peroxisome proliferator WY-14 643 in rats. It was hypothesized that the effect of glycine in these in vivo models was due to inhibition of proliferation of endothelium, thus preventing neovascularization and growth of tumors.
Glycine inhibits endothelial cell proliferation and migration

Rose et al. showed that dietary supplementation with glycine was shown to slow growth of B16 melanoma cells transplanted to the mouse and artery formation in tumors. Proliferation of endothelial cells is a key step in the process by which new blood vessels grow from established ones, and it was suggested that glycine may inhibit angiogenesis by preventing endothelial cell proliferation. To test the hypothesis that antiproliferative effects of the nutrient glycine observed in a B16 melanoma tumor model are due to inhibition of growth-factor-dependent proliferation and migration of endothelial cells, thus slowing neovascularization of the tumor, a series of experiments were performed. Bovine endothelial (CPA) cells were cultured in MEM media supplemented with 10% fetal bovine serum. For proliferation assays, cells were trypsinized, seeded onto 60-mm tissue culture dishes at a density of 5 x 10^5 cells per dish and cultured in MEM. After 24 h medium was changed and cells were incubated in the presence or absence of glycine (10 mmol/L) with or without VEGF (1 ng/mL). After 6 days, cells were trypsinized and counted. The number of endothelial cells was increased to approximately eightfold after 6 days. Moreover, addition of VEGF accelerated the proliferation of cells. Significant differences between groups in the presence and absence of VEGF were observed. However, addition of glycine blunted cell growth. Moreover, glycine also produced an inhibited VEGF effect completely.

Furthermore, the migration of endothelial cells was quantified using a Transwell culture chamber (Costar, Cambridge, MA, USA). Glycine added to culture media inhibited serum-induced migration of endothelial cells by approximately 50%. Glycine inhibited both endothelial cell proliferation and migration. These data suggest that glycine suppresses neovascularization of tumors, at least partially, by inhibiting endothelial cell proliferation and migration.

Glycine blunted increases in intracellular calcium concentration due to VEGF

The VEGF-induced proliferation and migration of endothelial cells, a central process in tumor angiogenesis, is dependent upon intracellular Ca^{2+}. To evaluate glycine effect on calcium signal, intracellular Ca^{2+} concentration was measured. For measurements of intracellular Ca^{2+}, cells were cultured for 24 h after plating. Intracellular Ca^{2+} concentration was measured fluorometrically using the fluorescent Ca^{2+}-indicator dye fura-2 and a microspectrofluorometer. Addition of VEGF (1 ng/mL) to endothelial cells increased intracellular Ca^{2+} concentration levels quickly to 141 ± 11 nmol/L from a basal level of 10 ± 1 nmol/L, followed by a rapid return to basal levels within 3 min. Glycine (1 mmol/L), when added before VEGF to CPA cells cultured in a glycine-free media, abolished the increases in intracellular Ca^{2+} concentration due to VEGF.

Glycine acts as an inhibitory neurotransmitter in the central nervous system via activation of a glycine-gated chloride channel that increases in intracellular Cl^- concentration and hyperpolarizes the plasma membrane. To test the hypothesis that the inhibitory effect of glycine on VEGF-stimulated increases in intracellular Ca^{2+} concentration in endothelial cells occurs via similar mechanisms, low concentrations of strychnine (1 μmol/L), antagonist of the glycine receptor, were added before glycine. Indeed, the inhibitory effect of glycine on the elevation of intracellular Ca^{2+} concentration by VEGF was blocked by 1 μmol/L strychnine. Second, cultured endothelial cells were incubated in chloride-free buffer where chloride was substituted by equimolar amounts of gluconate for 2 min before the addition of glycine. Under these conditions, the VEGF-induced increases in intracellular Ca^{2+} concentration were not inhibited by glycine. Thus, the inhibitory effect of glycine was dependent on extracellular chloride.

Endothelial cells contain glycine-gated chloride channel

Hyperpolarization of the plasma membrane due to influx of chloride is the mechanism of the inhibitory effect of glycine in the central nervous system and in a variety of white blood cells. To investigate whether glycine affects movement of chloride ions from the extracellular to the intracellular space, endothelial cells were incubated with ^36Cl in glycine-free media and glycine (1 mmol/L) was added. Glycine increased ^36Cl influx into endothelial cells approximately 10-fold, an effect that was nearly completed blocked by strychnine.

Glycine acts by binding to receptors that are localized largely in post-synaptic neuronal membranes. Glycine receptors in the neuron consist of three distinct protein subunits: a 48-kDa alpha subunit, a 58-kDa beta subunit, and a 93-kDa receptor-associated cytoplasmic protein. To determine if endothelial cells express glycine receptor, reverse transcription-polymerase chain reaction

![Figure 1](image-url) Working hypothesis for the mechanism of action of glycine in endothelial cells. It is proposed that vascular endothelial growth factor (VEGF) activates VEGF receptor, resulting in increases in intracellular calcium. Glycine blunts VEGF-induced increases in intracellular calcium concentration, an effect reversed by low concentration of strychnine and depletion of extracellular chloride. Inhibition of proliferation and migration by glycine is most likely due to the blunting of the calcium signaling pathway. These data are consistent with the hypothesis that glycine activates a glycine receptor, leading to the influx of chloride, which hyperpolarizes the endothelial cell membrane and decreases the opening time of voltage-dependent calcium channels. In vivo treatment with glycine blocks tumor growth and artery formation in tumors likely via this mechanism. ER, endoplasmic reticulum.
(RT-PCR) was performed. The RT-PCR amplification of endothelial cell mRNA revealed a 124-bp fragment as predicted from the known sequence of the spinal cord glycine receptor β-subunit. Furthermore, the PCR-amplified fragment was purified, sequenced and found to be 92% similar to the β-subunit of the spinal cord glycine receptor.

These data suggest that endothelial cells contain glycine-gated chloride channels.

**Conclusion**

Based on the data presented here, the following mechanism is proposed to explain these findings (Fig. 1). The VEGF activates VEGF receptor, which stimulates phospholipase C and increases intracellular calcium, a critical signal in proliferation and migration of endothelial cells. Glycine blunts VEGF-induced increases in intracellular calcium concentration, an effect reversed by low concentration of strychnine and depletion of extracellular chloride. Inhibition of proliferation and migration by glycine is most likely due to the blunting of calcium signals. These data are consistent with the hypothesis that glycine activates a glycine receptor, leading to the influx of chloride, which hyperpolarizes the endothelial cell membrane and decreases the opening time of voltage-dependent calcium channels. In vivo treatment with glycine blocks tumor growth and artery formation in tumors likely via this mechanism.

Therefore, glycine can be used not only for treatment of inflammation, but also for chemoprevention and treatment of carcinoma.

**References**