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(13) 肝類洞内皮細胞のアポトーシスの制御機構に関する研究

## 肝類洞内皮細胞のアポトーシスの制御機構に関する研究

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## 要旨

肝類洞内皮細胞は種々の肝疾患においてその早期から障害を受けることが知られている。近年肝阻血再灌流障害や肝移植時に肝類洞内皮細胞がアポトーシスに陥ることが明らかにされた。また、アミノ酸の1種であるグリシンには細胞保護作用があることが知られているが、近年、移植肝をグリシンで処置することにより、グラフト生着率が向上することが報告され注目されている。一方、肝類洞内皮細胞の単離および初代培養の系が確立され、この細胞の増殖のみならず生存に各種増殖因子、とくに Vascular Endothelial Growth Factor (VEGF) が必須であることが明らかとなった。本研究の目的は類洞内皮細胞の増殖因子枯渇時に生じる細胞死のメカニズムを検討し、さらに細胞死を制御する方法を検討することにある。(方法) Wistar 系雄性ラットよりコラゲナーゼ灌流および Percoll による比重遠心法で肝類洞内皮細胞を単離し、牛胎児血清および VEGF 含む各種増殖因子を添加した EBM-2 培地を用いて type 1 collagen coated dish 上で3日間培養した後、実験に供した。培地を VEGF 非添加 EBM-2 に交換し、4~12時間後に細胞を固定し、terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) 法を用いてアポトーシスの検出を行った。さらに、この類洞内皮細胞アポトーシスに対する各種アミノ酸の効果および機序について検討した。また、この際の類洞内皮細胞の Bcl-2 蛋白の変化を Western blot 法で確認した。(結果および考察) 1、まず、VEGF 枯渇時の類洞内皮細胞の形態変化を位相差顕微鏡で観察した。培養三日目の類洞内皮細胞は、紡錘状に伸展した形態をしている。しかしその後 VEGF を抜いた培地に交換してインキュベートすると細胞の伸展は阻害され dish から剥離する傾向を認めた。従って VEGF 枯渇によって類洞内皮細胞に細胞死が惹起されるものと考えられた。2、この類洞内皮細胞の VEGF 枯渇時の変化がアポトーシスであるかどうかを検討するために、TUNEL 法を用いて染色した。VEGF 存在下の類洞内皮細胞では TUNEL 陽性細胞はわずかに認められるのみであったが、VEGF 枯渇後4時間で TUNEL 陽性細胞の増加を認め、16時間以降はほとんどすべての細胞が TUNEL 陽性となった。濃染している核には凝縮や分断化が認められ、これらの細胞の大半がアポトーシスに陥っているものと考えられた。3、次に VEGF 枯渇によるアポトーシスに対するグリシンの影響を検討するために VEGF 枯渇と同時に培地中に1mM~10mM のグリシンを添加し、TUNEL 染色を試みた。グリシンは VEGF 枯渇によるアポトーシスを有意に抑制した。4、この VEGF 枯渇によってアポトーシスの抑制作用は、グリシンに特有のものであるかどうかを検討するために、他のアミノ酸(アラニン、バリン、グルタミン、メチオニン、セリン)を用いて同様に実験を行った。これらの他のアミノ酸には、アポトーシス抑制作用が認められなかった。すなわちアポトーシスの抑制作用はグリシンに特有のものと考えられた。5、グリシンには様々な作用があることが知られており、その一つとしてレセプターを介した反応があることが主に中枢神経系で知られている。ここで認められたグリシンのアポトーシス抑制作用がグリシンレセプターを介した反応であるかどうかを明らかにするために、グリシンレセプターの特異的アンタゴニストであるストリキニーネを用いて

検討した。グリシンのアポトーシス抑制効果は $1\mu\text{M}$  のストリキニーネによって有意に阻害されることが明らかとなった。つまり、グリシンのアポトーシス抑制効果の少なくとも一部にはグリシンレセプターを介した機序が関与している可能性があると考えられた。6、さらに、VEGF 枯渇時のアポトーシス関連蛋白の変動について検討した。他の血管内皮細胞を用いた検討では VEGF がアポトーシス抑制蛋白である Bcl-2 をアップレギュレートすることが報告されている。そこで類洞内皮細胞の VEGF 枯渇時による Bcl-2 の蛋白レベルがグリシンにより変化するかどうかを Western blot 法で検討した。VEGF 枯渇時には Bcl-2 が減少することおよびグリシンは VEGF 枯渇による Bcl-2 減少を抑制する作用を有することが明らかになった。

以上の検討より、グリシンが類洞内皮細胞のアポトーシスを抑制することによって肝移植時などにおける阻血再灌流障害を軽減する可能性が示唆された。

## KEY WORDS

cell death, glycine receptors, strychnine, chloride channels, Bcl-2

## OBJECTIVES

The aim of this study was: (1) to determine the death pattern of primary cultured sinusoidal endothelial cells (SECs) due to VEGF deprivation; (2) to investigate the effect of glycine on VEGF deprivation-induced apoptosis in SECs; and (3) to explore mechanism by which glycine prevents apoptosis in SECs.

## METHODS

### Isolation and primary culture of Liver Sinusoidal Endothelial Cells:

SECs were isolated from male Wister rats weighing 350 to 400g (Charles River Japan Inc. Saitama, Japan) by collagenase perfusion and differential centrifugation using Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the method described by F. Braet et al with slight modifications.<sup>1</sup> Briefly, the liver was perfused *in situ* through the portal vein with Gey's balanced salt solution (GBSS) without calcium at 37°C for 10 minutes at a flow rate of 20 mL/min, followed by GBSS with calcium containing 0.05% collagenase A (Roche Diagnostics Co., Mannheim, Germany) at 37°C for 10 minutes at a flow rate of 10 mL/min. Subsequently the liver was excised and further digested by shaking in 100 mL fresh GBSS with calcium containing 0.05% collagenase A, 0.001% DNase (Roche Diagnostics Co.) and 5.3 mL of heat-inactivated fetal bovine serum (FBS) for 5 minutes at 37°C. The cell suspension was then filtered through nylon gauze and centrifuged at 100g for 4 minutes. The supernatant enriched in SECs was centrifuged for 6 minutes at 350g, and then the pellet was layered on the double layer of Percoll gradient (25% and 50%) and centrifuged immediately at 900g for 20 minutes. The fraction of SECs was collected and washed in phosphate-buffered saline (PBS). The viability of SECs judged by the Trypan blue exclusion test was above 95% in all isolations. The purity of SECs examined by a phase contrast microscope was 89.45%±3.46%. Furthermore, the purity of cultured SECs was confirmed by the uptake of acetylated low-density lipoprotein and ovalbumin (data not shown). Cells were seeded onto collagen-coated polyethylene dishes (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and cultured in EBM-2 medium (Bio Whittaker Co., Walkersville, MD), supplemented with the EGM-2 bullet kit (Bio Whittaker Co.; containing long R insulin-like growth factor-1, human epidermal growth factor, human fibroblast growth factor-B) and 20ng/mL human recombinant VEGF (Wako Junyaku Co., Osaka, Japan) in humidified air with 5% CO<sub>2</sub> at 37°C for 3 days before experiments.

### Induction of Cell Death in Primary cultured SECs and Evaluation of Apoptosis:

At day 3 of culture, cell death of primary cultured SECs was induced by exchanging the culture media with VEGF-free EBM-2 (VEGF deprivation). Cells were then further cultured in a CO<sub>2</sub> incubator for up to 24 hours. Morphological changes of SECs were observed and photographed under a phase-contrast microscope (F-601 AF Quartz Data, Nikon Optics, Tokyo, Japan).

Apoptosis of SECs was detected by a terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) technique using a fluorescein apoptosis detection system kit (Apoptosis Detection System, Fluorescein, Promega Corp., Madison, WI) according to the manufacturer's instructions. This method measures the fragmented DNA by catalytically incorporating fluorescein-12-dUTP at the 3'-OH ends generated by DNA fragmentation using the TdT enzyme. Briefly, cells were fixed with 4% formalin, permeabilized with 0.2% Triton X-100 in PBS on ice, and incubated in an equilibration buffer containing 5  $\mu$ mol/L fluorescein-12-dUTP and 25 U TdT for 1 hour at 37°C to stain fragmented DNA. Subsequently, 0.2  $\mu$ g/mL propidium iodide in PBS was applied for 15 minutes to stain both apoptotic and nonapoptotic cells. Thus, propidium iodide stains both apoptotic and nonapoptotic cells red throughout the cytoplasm, whereas fluorescein-12-dUTP is incorporated at the 3'-OH ends of fragmented DNA, resulting in localized green fluorescence within the nucleus of apoptotic cells. The stained cells were observed under a fluorescence microscope (Axiophot ZVST<sup>TM</sup> 3C75DE, Carl Zeiss Inc, Goleta, CA) and the photographs were taken. To calculate the percentage of apoptotic SECs, TUNEL-positive nuclei were counted in a middle-power field (200 $\times$ ). Four random fields were examined in a blinded manner in each preparation.

### Western Blotting for Bcl-2:

Cultured SECs were washed with ice-cold PBS and harvested by scraping in cold PBS, followed by a centrifugation at 1,500 rpm for 5 minutes. The cell pellet was then homogenized in a buffer containing 66 mmol/L Tris, pH 8.0; 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton

X-100, and protease/phosphatase inhibitors [40  $\mu$ g/mL Bestatin (Roche Diagnostics Co.), 20 mmol/L  $\beta$ -glycerophosphate (Sigma Chemical Co., St. Louis, MO), 10 mmol/L p-nitrophenyl phosphate (PNPP; Roche Diagnostics Co.), 500  $\mu$ mol/L Pefabloc (Roche Diagnostics Co.), 700 ng/mL Pepstatin A (Roche Diagnostics Co.), 2  $\mu$ g/mL Aprotinin (Roche Diagnostics Co.), 500  $\mu$ mol/L Na<sub>3</sub>VO<sub>4</sub> (Sigma Chemical), and 500 ng/mL Leupeptin (Roche Diagnostics Co.)]. After centrifugation at 15,000 rpm for 15 minutes, the supernatant was collected and protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots containing equal amounts of proteins were suspended in sodium dodecyl sulfate (SDS)-glycerol loading buffer (62.5 mmol/L Tris pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue), and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA) using a semidry transfer apparatus. Membranes blocked with 20% FBS in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for nonspecific bindings of antibodies were hybridized with a polyclonal rabbit anti-Bcl-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 20% FBS in TBS-T for 2 hours at room temperature, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc.) in 20% FBS in TBS-T for 1 hour at room temperature. An enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech, Denver, CO) was applied for the detection of specific bands according to the manufacturer's instructions.

#### **Statistical Analysis:**

Data were expressed as mean  $\pm$ SD, and analysis of variance (ANOVA) and the Tukey's post-hoc test were used for the multiple comparisons of the differences in mean values.  $P < 0.05$  was selected before the analysis to determine the significance.

## **RESULTS**

### **VEGF deprivation causes apoptosis in primary cultured rat SECs:**

Under a phase contrast microscope, the primary cultured rat SECs showed spindle-like shapes and were nearly confluent at 3 days of culture. However, cells tended to shrink during the time course of VEGF deprivation, and finally, cells started detaching from dishes in 24 hours of VEGF deprivation, indicating that VEGF deprivation caused cell death in SECs.

To clarify whether the cell death of SECs as a result of VEGF deprivation is apoptosis, TUNEL staining was performed. As expected, most cells 3 days after culture in EBM-2 medium containing VEGF were negative for TUNEL staining, and TUNEL-positive cells were only 2.0% in this condition. However, the TUNEL-positive cells start to increase in number 4 hours after VEGF deprivation, and almost all cells were TUNEL-positive 16 hours after VEGF deprivation. The nuclei of TUNEL-positive cells showed condensation and fragmentation under a high-power view, representing typical features of apoptotic cells (data not shown).

### **Effects of Glycine on VEGF deprivation-induced apoptosis of SECs:**

To evaluate the protective effect of amino acids against the apoptosis of SECs, we utilized this VEGF deprivation-treated apoptosis in primary cultured SECs. Interestingly, this increase in TUNEL-positive cells after VEGF deprivation was significantly prevented by addition of glycine (1-10 mmol/L) in the medium in a dose dependent manner. The percentages of apoptotic cells in the presence of glycine were almost half 4 hours and 8 hours after VEGF deprivation. To investigate whether this antiapoptotic effect on SECs is glycine-specific, the effects of other amino acids were examined. The percentages of apoptotic cells 8 hours after VEGF deprivation were  $59.80\% \pm 13.37\%$ ,  $65.96\% \pm 10.40\%$ ,  $60.53\% \pm 7.95\%$ ,  $62.11\% \pm 4.98\%$  and  $71.27\% \pm 15.16\%$  in the presence of 10 mmol/L alanine, valine, glutamine, methionine and serine, respectively, which had no significant difference as compared with VEGF-deprived controls. These observations indicated that the amino acids other than glycine tested here had no protective effect on the apoptosis in primary cultured SECs, and the effect of glycine was unique.

### **Strychnine Antagonizes the Effect of Glycine on the Apoptosis in SECs:**

To test the hypothesis that this antiapoptotic effect of glycine in SECs is mediated through glycine receptor on the plasma membrane, we tried strychnine, a putative glycine receptor antagonist<sup>2</sup>. The percentage of TUNEL-positive cells 8 hours after VEGF deprivation was  $40.3\% \pm 2.0\%$  when strychnine (1  $\mu$ mol/L) was added in combination with glycine (10 mmol/L),

which was significantly higher than that of glycine alone ( $P < 0.05$ ). In contrast, the percentage of TUNEL-positive cells 8 hours after VEGF deprivation in the presence of strychnine ( $1 \mu\text{mol/L}$ ) was  $50.8\% \pm 9.3\%$ , which was not significantly different from VEGF deprivation alone. These findings indicated that a low dose of strychnine inhibited, at least in part, the antiapoptotic effect of glycine in primary cultured SECs.

#### **Bcl-2 Expression in SECs Under VEGF Deprivation:**

The expression of Bcl-2, an antiapoptotic protein, in SECs was detected by Western blotting. As expected, the 25- to 26-kd specific band for Bcl-2 was detected clearly in the control SECs 3 days after inoculation. However, the expression of Bcl-2 was decreased dramatically in the SECs 8 hours after VEGF deprivation. Surprisingly, the decrease of Bcl-2 as a result of VEGF deprivation was prevented almost completely in the presence of  $10 \text{ mmol/L}$  glycine.

### **DISCUSSION**

Necrosis is a passively uncontrolled process and usually occurs in large cell groups, which characterized morphologically by cell swelling and bursting, nuclear and cytoplasmic lysis. However, apoptosis (programmed cell death) is a tightly controlled process and typically occurs in single cell, which characterized morphologically by cell shrinkage, nuclear and cytoplasmic blebbing, disintegration of nuclear membrane and formation of apoptosis bodies. The activation of the *de novo* synthesis of endonucleases results in fragmentation of DNA, which can be detected by TUNEL method. Deprivation of growth factors is well known to induce apoptosis in various types of cells. In the current study, TUNEL-positive SECs increased dramatically during the time course of VEGF deprivation, and the TUNEL-positive nuclei showed condensation and fragmentation in shape, which are characteristic morphological features of apoptosis. According to the characteristics of the TUNEL positive nuclei, we demonstrates that VEGF deprivation caused apoptotic cell death in primary cultured SECs, which constitutes a promising system for studying the protective chemicals of apoptosis of SECs.

It was reported that SECs express VEGF receptors (Flt-1 and KDR/Flk-1) abundantly, and VEGF maintains isolated SECs for an extended time course in culture, indicating that VEGF is one of the essential survival factors for SECs<sup>3</sup>. More recently, Moriga et al. reported that VEGF is preventive against sinusoidal endothelial damage and apoptosis induced by cold ischemia-reperfusion.<sup>4</sup> These findings support the hypothesis that VEGF plays a crucial role in the damage of SECs during cold ischemia-reperfusion, including liver transplantation. Therefore, we evaluated the effect of glycine on apoptotic cell death in SECs induced by VEGF deprivation. Interestingly, glycine prevented this VEGF deprivation-induced apoptosis in SECs in a dose-dependent manner. Furthermore, the other amino acids we tested (alanine, valine, glutamine, methionine, and serine) had no effect in this model, which indicated that this antiapoptotic effect of glycine is a unique property rather than a nonspecific effect of amino acids. It is concluded, therefore, that glycine has an antiapoptotic effect in SECs.

It is well known that glycine acts as a neurotransmitter in the central nervous system, and a glycine receptor has been identified and well characterized. The glycine receptor in the central nervous system constitutes a chloride channel on the plasma membrane, and glycine causes the influx of chloride ion, leading to hyperpolarization of the plasma membrane, thereby counteracting depolarization stimuli.<sup>5</sup> Recently, Kupffer cells, resident macrophages in the liver, have been demonstrated to possess a similar type of receptors, by which glycine prevents the production of inflammatory cytokine.<sup>6</sup> These observations suggest a possibility that SECs contain a similar type of glycine receptor on the plasma membrane. To clarify whether the antiapoptotic effect of glycine observed here is through this possible glycine receptors, strychnine, a putative glycine receptor antagonist, was tested. Interestingly, the antiapoptotic effect of glycine was antagonized in part by a low dose of strychnine ( $1 \mu\text{mol/L}$ ). It is postulated, therefore, that SECs most likely contain a glycine receptor, though which glycine elicits antiapoptotic properties. It is not clear how the glycine receptor modulates apoptotic cell death, but one possibility is that this glycine receptor/chloride channel increased the chloride influx, which may alter the osmolarity of cytoplasm, thereby preventing the shrinkage of cells during the apoptotic process.

On the other hand, Bcl-2, an antiapoptotic protein locating on the mitochondrial membrane,<sup>7</sup> is recognized as one of the key factors in growth factor deprivation-induced apoptosis in endothelial cells. Indeed, it has been reported that VEGF increases the expression of Bcl-2 in human umbilical



vein endothelial cells, and the overexpression of Bcl-2 prevents the apoptotic cell death caused by growth factor deprivation in human umbilical vein endothelial cells.<sup>8</sup> Furthermore, the adenoviral gene transfer of Bcl-2 has been shown to reduce ischemia-reperfusion injury in the liver.<sup>9</sup> In the present study, it was demonstrated that Bcl-2 protein levels decreased dramatically after 8 hours of VEGF deprivation in isolated SECs, which may be one of the key regulating factors of apoptosis under this condition. Surprisingly, glycine prevented this decrease of Bcl-2 protein levels almost completely. Although the precise mechanisms by which glycine prevented the decrease of Bcl-2 is unclear, these findings support the hypothesis that glycine prevents apoptosis of SECs by maintaining Bcl-2 protein levels.

In conclusion, glycine is protective against apoptotic cell death in SECs. Glycine most likely acts through glycine receptor on SECs, and it also prevents the decrease of Bcl-2 under VEGF deprivation. This antiapoptotic effect of glycine in SECs might be involved, at least in part, in the mechanism by which glycine prevents various types of liver injury in which apoptosis of SECs plays an important role.

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# Glycine Prevents Apoptosis of Rat Sinusoidal Endothelial Cells Caused by Deprivation of Vascular Endothelial Growth Factor

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Apoptosis of sinusoidal endothelial cells (SECs) is one of the initial events in the development of ischemia-reperfusion injury of the liver. Glycine has been shown to diminish ischemia-reperfusion injury in the liver and improve graft survival in the rat liver transplantation model. Here, we investigated the effect of glycine on apoptosis of primary cultured rat SECs induced by vascular endothelial growth factor (VEGF) deprivation. Isolated rat SECs were cultured in EBM-2 medium supplemented with 10% fetal bovine serum (FBS) and growth factors including 20 ng/mL VEGF for days. SECs at 3 days of culture showed spindle-like shapes; however, cells started shrinking and detaching from dishes by VEGF deprivation. Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining in these conditions. Control SECs contained only a few percent of TUNEL-positive cells; however, they started increasing 4 hours after VEGF deprivation, and the percentage of TUNEL-positive cells reached about 50% at 8 hours and almost 100% at 16 hours after VEGF deprivation. Interestingly, this increase in TUNEL-positive cells after VEGF deprivation was prevented significantly when glycine (1–10 mmol/L) was added to the medium, the levels being around 60% of VEGF deprivation without glycine. Furthermore, strychnine (1  $\mu$ mol/L), a glycine receptor antagonist, inhibited this effect of glycine, suggesting the possible involvement of the glycine receptor/chloride channel in the mechanism. Moreover, Bcl-2 protein levels in SECs were decreased 8 hours after VEGF deprivation, which was prevented almost completely by glycine. It is concluded that glycine prevents apoptosis of primary cultured SECs under VEGF deprivation. (HEPATOLOGY 2000;32:542–546.)

Liver sinusoidal endothelial cells (SECs) have been demonstrated to play an important role in the development of various types of liver injury. In particular, the cell death of SECs is one of the initial events in ischemia-reperfusion injury in the

liver,<sup>1</sup> and the degrees of injury in SECs have inversely correlated well with the graft survival in liver transplantation. Recently, several reports demonstrated that apoptosis occurs primarily in SECs after cold storage–reperfusion of the liver,<sup>2–4</sup> indicating that the apoptosis of SECs most likely plays a pivotal role in primary graft nonfunction. In this aspect, prevention of apoptosis in SECs appears to be important for successful liver transplantation. On the other hand, vascular endothelial growth factor (VEGF) is one of the key factors for the survival and proliferation of endothelial cells.<sup>5</sup> Indeed, the starvation of serum or growth factor has been used for the induction of apoptosis in various types of endothelial cells in culture. It was also reported that VEGF promotes extended survival of SECs in the primary culture.<sup>6</sup>

Glycine, a nonessential amino acid, is known to possess various physiological functions besides being a source of protein synthesis and metabolism. In the central nervous system, glycine works as a neurotransmitter, and a glycine-receptor is identified in nerve cells.<sup>7</sup> Furthermore, cyto- and organ-protective effects of glycine has been reported in renal tubules,<sup>8–10</sup> isolated hepatocytes,<sup>11–13</sup> and an ischemia-reoxygenation injury model using perfused liver *ex vivo*.<sup>14</sup> It has also been demonstrated that a diet containing glycine is preventive against endotoxin-induced mortality and liver injury<sup>15</sup> and early alcohol-induced liver injury in the rat.<sup>16</sup> Moreover, the organ rinse solution containing glycine increased graft survival in the rat liver transplantation model,<sup>17</sup> and intravenous administration of glycine to the recipient also improved survival in rat liver transplantation.<sup>18</sup> The effect of glycine rinse on cold ischemia-reperfusion injury in the liver was further confirmed in human liver transplantation.<sup>19</sup> More recently, Nishimura et al. reported that hypoxia-reoxygenation injury in isolated SECs was reduced by glycine.<sup>20</sup> These observations support the hypothesis that glycine is cytoprotective in various types of cells including SECs; however, it is not clear whether glycine can prevent apoptotic cell death in SECs. Therefore, the purpose of this study was to investigate the effect of glycine on VEGF deprivation–induced apoptosis in primary cultured SECs. Furthermore, we investigated the mechanism by which glycine prevents apoptosis in SECs. The preliminary account of this work has been reported elsewhere in abstract form.<sup>21</sup>

## MATERIALS AND METHODS

**Isolation and Primary Culture of Liver Sinusoidal Endothelial Cells.** SECs were isolated from male Wistar rats weighing 350 to 400 g (Charles River Japan Inc., Saitama, Japan) by collagenase perfusion and differential centrifugation using Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the method described by Braet et al., with some modifications.<sup>22</sup> All animals used in this study received humane care, and the protocol was approved by the commit-

Abbreviations: SEC, sinusoidal endothelial cell; VEGF, vascular endothelial growth factor; GBSS, Gey's balanced salt solution; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotidyl transferase; dUTP, d-uridine triphosphate; TUNEL, TdT-mediated dUTP-biotin nick end labeling; TBS, Tris-buffered saline.

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tee of laboratory animals according to institutional guidelines. Briefly, the liver was perfused *in situ* through the portal vein with Gey's balanced salt solution (GBSS) without calcium at 37°C for 10 minutes at a flow rate of 20 mL/min, followed by GBSS with calcium containing 0.05% collagenase A (Roche Diagnostics Co., Mannheim, Germany) at 37°C for 10 minutes at a flow rate of 10 mL/min. Subsequently, the liver was excised and further digested by shaking in 100 mL fresh GBSS with calcium containing 0.05% collagenase A, 0.001% DNase (Roche Diagnostics Co.), and 5.3 mL heat-inactivated fetal bovine serum (FBS) for 5 minutes at 37°C. The cell suspension was then filtered through nylon gauze and centrifuged at 100g for 4 minutes. The supernatant enriched in SECs was centrifuged for 6 minutes at 350g, and then the pellet was layered on the double layer of Percoll gradients (25% and 50%) and centrifuged immediately at 900g for 20 minutes. The fraction of SECs was collected and washed in phosphate-buffered saline (PBS). The viability of isolated SECs judged by the Trypan blue exclusion test was above 95% in all isolations. The purity of SECs examined by a phase-contrast microscope was  $89.5\% \pm 3.5\%$ . Furthermore, the purity of cultured SECs was confirmed by the uptake of acetylated low-density lipoprotein and ovalbumin (data not shown). Cells were seeded onto collagen-coated polyethylene dishes (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and cultured in EBM-2 medium (Bio Whittaker Co., Walkersville, MD), supplemented with the EGM-2 bullet kit (Bio Whittaker Co.; containing long R insulin-like growth factor-1, human epidermal growth factor, and human fibroblast growth factor-B) and 20 ng/mL human recombinant VEGF (Wako Junyaku Co., Osaka, Japan) in humidified air with 5% CO<sub>2</sub> at 37°C for 3 days before experiments.

**Induction of Cell Death in Primary Cultured SECs and Evaluation of Apoptosis.** At day 3 of culture, cell death of primary cultured SECs was induced by deprivation of VEGF by exchanging the culture media with VEGF-free EBM-2. Cells were then further cultured in a CO<sub>2</sub> incubator for up to 24 hours. Morphological changes of SECs were observed and photographed under a phase-contrast microscope (F-601 AF Quartz Data, Nikon Optics, Tokyo, Japan).

Apoptosis of SECs was detected by a terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) technique using a fluorescein apoptosis detection system kit (Apoptosis Detection System, Fluorescein, Promega Corp., Madison, WI) according to the manufacturer's instructions. Briefly, cells were fixed with 4% formalin, permeabilized with 0.2% Triton X-100 in PBS on ice, and incubated in an equilibration buffer containing 5  $\mu$ M fluorescein-12-dUTP and 25 U TdT for 1 hour at 37°C to stain fragmented DNA. Subsequently, 0.2  $\mu$ g/mL propidium iodide in PBS was applied for 15 minutes to stain both apoptotic and nonapoptotic cells. Thus, propidium iodide stains both apoptotic and nonapoptotic cells red throughout the cytoplasm, whereas fluorescein-12-dUTP is incorporated at the 3'-OH ends of fragmented DNA, resulting in localized green fluorescence within the nucleus of apoptotic cells. The stained cells were observed under a fluorescence microscope (Axiophot ZVS 3C75DE, Carl Zeiss Inc., Goleta, CA), and photographs were taken. To calculate the percentage of apoptotic SECs, TUNEL-positive cells were counted in a middle-power field (200 $\times$ ). Four random fields were examined in a blinded manner in each preparation.

**Western Blotting for Bcl-2.** Cultured SECs were washed with ice-cold PBS and harvested by scraping, followed by a centrifugation at 1,500 rpm for 5 minutes. The cell pellet was homogenized in a buffer containing 66 mmol/L Tris (pH 8.0), 5 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, and protease/phosphatase inhibitors (40  $\mu$ g/mL bestatin [Roche Diagnostics], 20 mmol/L  $\beta$ -glycerophosphate [Sigma Chemical Co., St. Louis, MO], 10 mmol/L *p*-nitrophenyl phosphate [Roche Diagnostics], 500  $\mu$ mol/L Pefabloc [Roche Diagnostics], 700 ng/mL pepstatin A [Roche Diagnostics], 2  $\mu$ g/mL aprotinin [Roche Diagnostics], 500  $\mu$ mol/L Na<sub>3</sub>VO<sub>4</sub> [Sigma Chemical], and 500 ng/mL leupeptin [Roche Diagnostics]). After centrifugation at 15,000 rpm for 15 minutes, the supernatant was collected and protein concentration was determined using a Bio-Rad protein

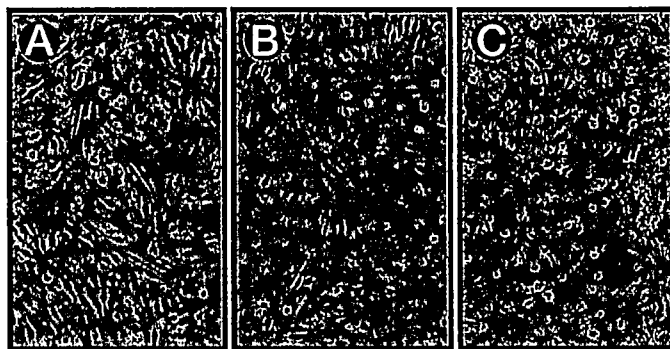


FIG. 1. Morphological changes in primary cultured rat SECs under VEGF deprivation. SECs were isolated from rat livers by collagenase digestion and cultured for 3 days before the experiment, as described in Materials and Methods. Cells were then cultured in fresh media without VEGF (VEGF deprivation) for 24 hours. Representative phase-contrast views of SECs from 5 individual experiments are shown. (A) Control SECs 3 days after inoculation. (B) SECs 12 hours after VEGF deprivation. (C) SECs 24 hours after VEGF. (Original magnification  $\times 200$ .)

assay kit (Bio-Rad Laboratories, Hercules, CA). The whole-cell protein extracts (20  $\mu$ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) using a semidry transfer apparatus. Membranes were blocked with 20% FBS in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for nonspecific bindings of antibodies, and then hybridized with a polyclonal rabbit anti-Bcl-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 20% FBS in TBS-T for 2 hours at room temperature. Membranes were washed 3 times with TBS for 5 minutes and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) in 20% FBS in TBS-T for 1 hour at room temperature. Following 3 $\times$  washes with TBS for 5 minutes, an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Denver, CO) was applied for the detection of specific bands according to the manufacturer's instructions.

**Statistical Analysis.** Data were expressed as mean  $\pm$  SD, and ANOVA and the Tukey's *post-hoc* test were used for the multiple comparisons of the differences in mean values.  $P < .05$  was selected before the analyses to determine the significance.

## RESULTS

**VEGF Deprivation Causes Apoptosis in Primary Cultured Rat SECs.** Under a phase-contrast microscope, the primary cultured rat SECs showed spindle-like shapes and were nearly confluent at 3 days of culture. However, cells tended to shrink during the time course of VEGF deprivation, and finally, cells started detaching from dishes after 24 hours of VEGF deprivation (Fig. 1), indicating that VEGF deprivation caused cell death in SECs. To clarify whether the cell death of SECs as a result of VEGF deprivation is apoptosis, TUNEL staining was performed (Fig. 2). As expected, most cells 3 days after culture in EBM-2 medium containing VEGF were negative for TUNEL staining, and TUNEL-positive cells were only 2.0% in this condition. However, the TUNEL-positive cells start to increase in number 4 hours after VEGF deprivation, and almost all cells were TUNEL-positive 16 hours after VEGF deprivation (Fig. 3). The nuclei of TUNEL-positive cells showed condensation and fragmentation under a high-power view, representing typical features of apoptotic cells (data not shown).

**Effects of Glycine on VEGF Deprivation-Induced Apoptosis of SECs.** To evaluate the protective effect of amino acids against the apoptosis of SECs, we used this VEGF deprivation-treated

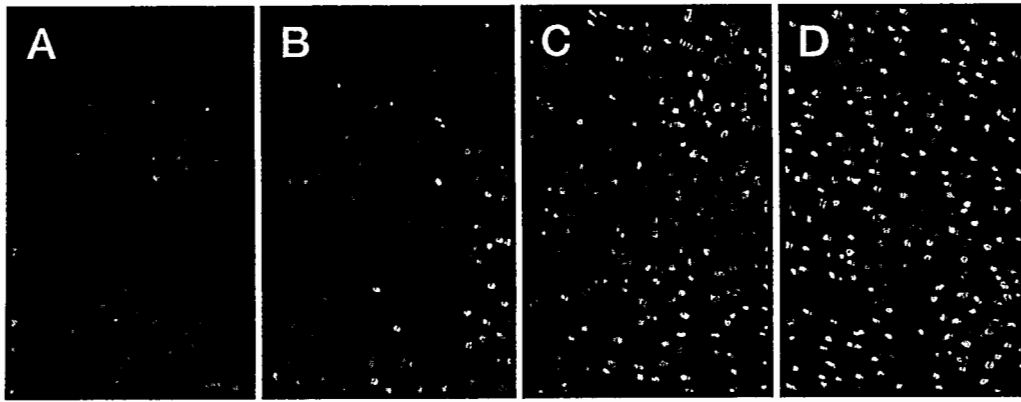


FIG. 2. Apoptosis of primary cultured rat SECs under VEGF deprivation. Experimental design as in Fig. 1. Apoptotic cells were detected by TUNEL staining as described in detail in Materials and Methods. Photographs were taken under fluorescent microscopy. (A) Control SECs 3 days after inoculation. (B) SECs 4 hours after VEGF deprivation. (C) SECs 8 hours after VEGF deprivation. (D) SECs 12 hours after VEGF deprivation. Representative photographs from 5 individual experiments are shown. (Original magnification  $\times 200$ .)

apoptosis in primary cultured SECs. Interestingly, the increase in TUNEL-positive cells as a result of VEGF deprivation was significantly prevented by the addition of glycine (1-10 mmol/L) in the medium in a dose dependent manner (Fig. 4). The percentages of apoptotic cells in the presence of glycine were almost half 4 hours and 8 hours after VEGF deprivation (Fig. 5). To investigate whether this antiapoptotic effect in SECs is glycine-specific, the effects of other amino acids were examined. The percentages of apoptotic cells 8 hours after VEGF deprivation were  $59.8\% \pm 13.4\%$ ,  $66.0\% \pm 10.4\%$ ,  $60.5\% \pm 8.0\%$ ,  $62.1\% \pm 5.0\%$ , and  $71.3\% \pm 15.2\%$  in the presence of 10 mmol/L alanine, valine, glutamine, methionine, and serine, respectively, which had no significant difference as compared with VEGF-deprived controls. These ob-

servations indicated that the amino acids other than glycine tested here had no protective effect on the apoptosis in primary cultured SECs, and the effect of glycine was unique.

**Strychnine Antagonizes the Effect of Glycine on the Apoptosis in SECs.** To test the hypothesis that this antiapoptotic effect of glycine in SECs is mediated through glycine receptors on the plasma membrane, we tried strychnine, a putative glycine receptor antagonist.<sup>23</sup> Figure 6 shows the percentage of TUNEL-positive cells 8 hours after VEGF deprivation in the presence of glycine (10 mmol/L) and strychnine (1  $\mu$ mol/L). The percentage of TUNEL-positive cells was  $40.3\% \pm 2.0\%$  when strychnine was added in combination with glycine, which was significantly higher than that of glycine alone ( $P < .05$ ). In contrast, the percentage of TUNEL-positive cells 8 hours after VEGF deprivation in the presence of strychnine alone (1  $\mu$ mol/L) was  $50.8\% \pm 9.3\%$ , which was not significantly different from VEGF deprivation alone. These findings indicated that a low dose of strychnine inhibited, at least in part, the antiapoptotic effect of glycine in primary cultured SECs.

**Bcl-2 Expression in SECs Under VEGF Deprivation.** The expression of Bcl-2, an antiapoptotic protein, in SECs was detected by Western blotting (Fig. 7). As expected, the 25- to 26-kd specific band for Bcl-2 was detected clearly in the control SECs 3 days after inoculation. However, the expression of Bcl-2 was decreased dramatically in the SECs 8 hours after VEGF deprivation. Surprisingly, the decrease of Bcl-2 as a

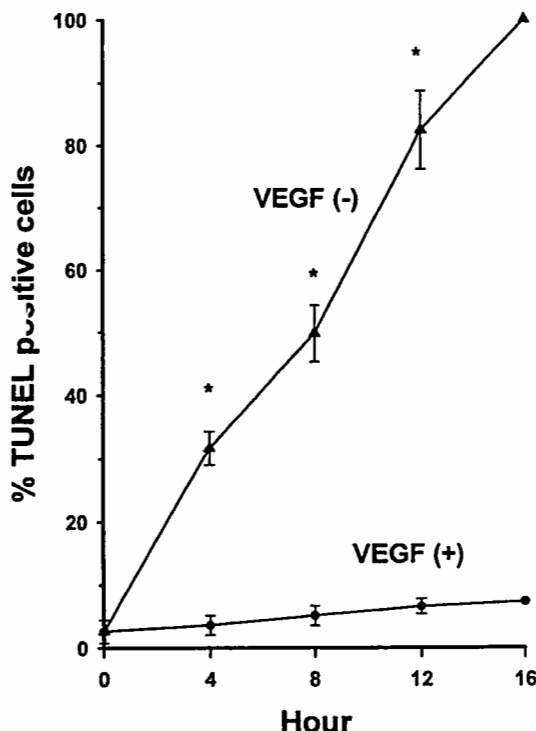


FIG. 3. Time course of VEGF deprivation-induced apoptosis in rat SECs. Experimental design as in Fig. 2. TUNEL-positive cells were counted in a middle-power field ( $\times 200$ ) in each condition, and the percentages of TUNEL-positive cells were plotted. Data represent mean  $\pm$  SD of 5 individual experiments (\* $P < .05$  by ANOVA and Tukey's post-hoc test as compared with controls).

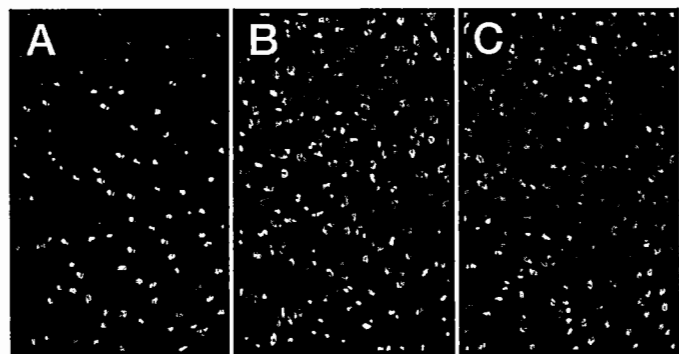


FIG. 4. Effect of glycine on VEGF deprivation-induced apoptosis in rat SECs. Experimental design as in Fig. 2. Glycine (1-10 mmol/L) was added in the medium without VEGF. (A) SECs 8 hours after VEGF deprivation (control). (B) SECs 8 hours after VEGF deprivation in the presence of 1 mmol/L glycine. (C) SECs 8 hours after VEGF deprivation in the presence of 10 mmol/L glycine. Representative photographs from 5 individual experiments are shown. (Original magnification  $\times 200$ .)

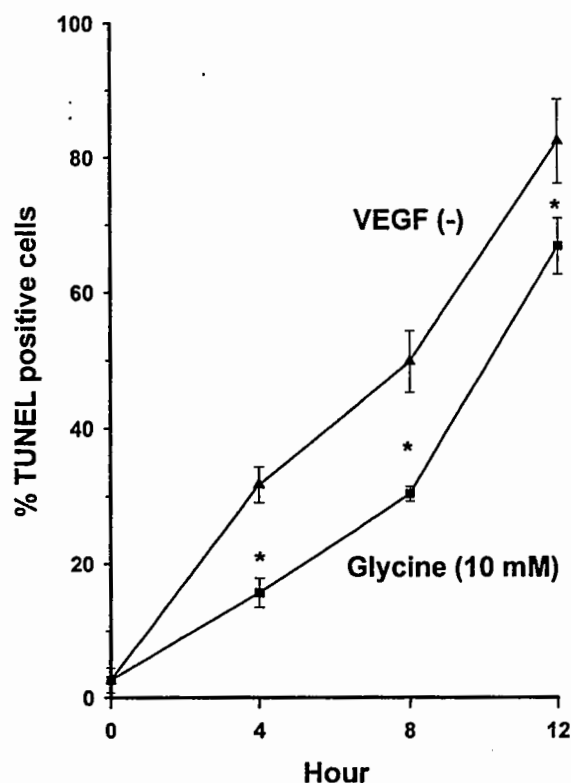


FIG. 5. Effect of glycine on VEGF deprivation-induced apoptosis in rat SECs. Experimental design as in Fig. 4. Percentages of TUNEL-positive cells were plotted. Data represent mean  $\pm$  SD of 5 individual experiments (\* $P$  < .05 by ANOVA and Tukey's *post-hoc* test as compared with VEGF-deprivation groups).

result of VEGF deprivation was prevented almost completely in the presence of 10 mmol/L glycine.

#### DISCUSSION

Deprivation of growth factors is well known to induce apoptosis in various types of cells. In this study, we demonstrated that VEGF deprivation caused apoptotic cell death in primary cultured SECs (Figs. 1 and 2). TUNEL-positive SECs increased dramatically during the time course of VEGF deprivation (Fig. 3), and the TUNEL-positive nuclei showed condensation and fragmentation in shape, which are characteristic morphological features of apoptosis. It was reported that SECs express VEGF receptors (Flt-1 and KDR/Flk-1) abundantly, and VEGF maintains isolated SECs for an extended time course in culture, indicating that VEGF is one of the essential survival factors for SECs.<sup>6</sup> More recently, Moriga et al. reported that VEGF is preventive against sinusoidal endothelial damage and apoptosis induced by cold ischemia-reperfusion.<sup>24</sup> These findings support the hypothesis that VEGF plays a crucial role in the damage of SEC during cold ischemia-reperfusion, including liver transplantation. Therefore, we evaluated the effect of glycine on apoptotic cell death in SECs induced by VEGF deprivation. Interestingly, glycine prevented this VEGF deprivation-induced apoptosis in SECs in a dose-dependent manner (Fig. 4). Furthermore, the other amino acids we tested (alanine, valine, glutamine, methionine, and serine) had no effect in this model, which indicated that this antiapoptotic effect of glycine is a unique property rather than a nonspecific effect of amino acids. It is concluded, therefore, that glycine has an antiapoptotic effect in SECs.

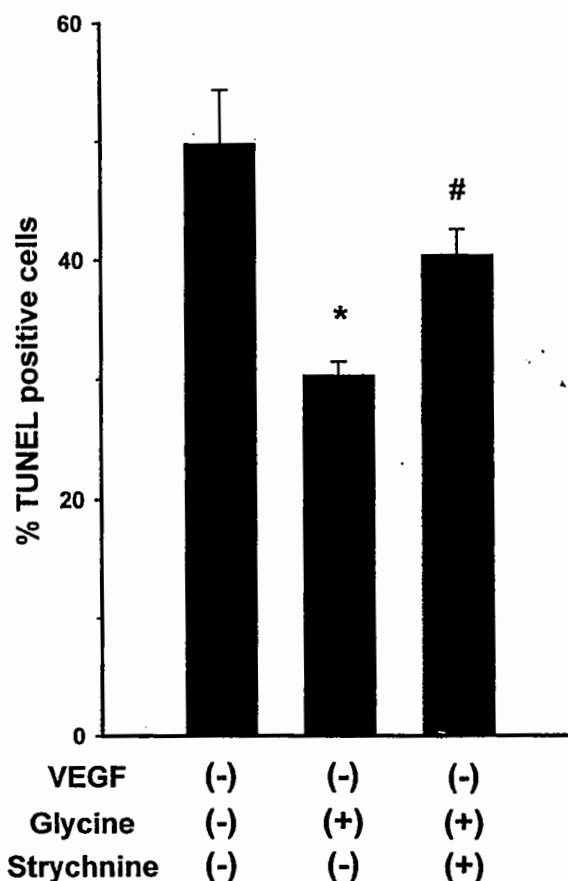


FIG. 6. Strychnine antagonizes the antiapoptotic effect of glycine in rat SECs. Experimental design as in Fig. 4. Strychnine (1  $\mu$ mol/L), a glycine receptor antagonist, was added to the media without VEGF supplemented with glycine (10 mmol/L), and cells were incubated for 8 hours before TUNEL staining. Percentages of TUNEL-positive cells were plotted. Data represent mean  $\pm$  SD from 5 individual experiments (\* $P$  < .05 as compared with controls; # $P$  < .05 as compared with glycine alone by ANOVA and Tukey's *post-hoc* test).

It is well known that glycine acts as a neurotransmitter in the central nervous system, and a glycine receptor has been identified and well characterized. The glycine receptor in the

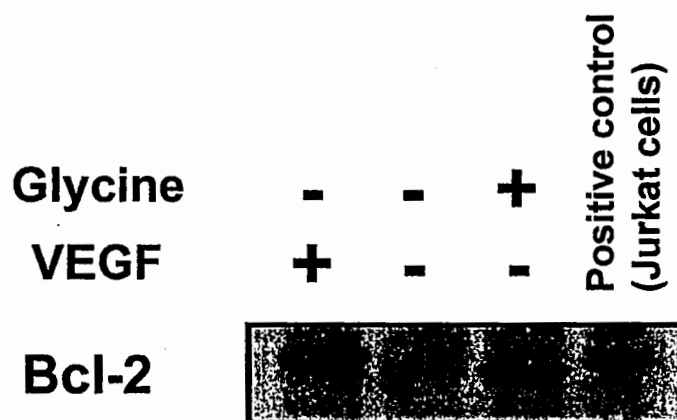


FIG. 7. Effect of glycine on Bcl-2 expression in rat SECs under VEGF deprivation. Bcl-2 in rat SECs was detected by Western blotting using a polyclonal antibody for Bcl-2 as described in Materials and Methods. Specific bands at 25 to 26 kd are shown. First lane: SECs 3 days after inoculation (control); second lane: SECs were incubated in the media without VEGF for 8 hours; third lane: SECs were incubated in the media without VEGF in the presence of glycine for 8 hours; fourth lane: whole-cell extracts from Jurkat cells (positive control).

central nervous system constitutes a chloride channel on the plasma membrane, and glycine causes the influx of chloride ion, leading to hyperpolarization of the plasma membrane, thereby counteracting depolarization stimuli.<sup>7</sup> Recently, Kupffer cells, resident macrophages in the liver, have been demonstrated to possess a similar type of receptors, by which glycine prevents the production of inflammatory cytokine production.<sup>25</sup> These observations suggest a possibility that SECs contain a similar type of glycine receptor on the plasma membrane. To clarify whether the antiapoptotic effect of glycine observed here is through this possible glycine receptors, strychnine, a putative glycine receptor antagonist, was tested (Fig. 6). Interestingly, the antiapoptotic effect of glycine was antagonized in part by a low dose of strychnine (1  $\mu$ mol/L). It is postulated, therefore, that SECs most likely contain a glycine receptor, through which glycine elicits antiapoptotic properties. It is not clear how the glycine receptor modulates apoptotic cell death, but one possibility is that this glycine receptor/chloride channel increased the chloride influx, which may alter the osmolarity of cytoplasm, thereby preventing the shrinkage of cells during the apoptotic process.

On the other hand, Bcl-2, an antiapoptotic protein locating on the mitochondrial membrane,<sup>26</sup> is recognized as one of the key factors in growth factor deprivation-induced apoptosis in endothelial cells. Indeed, it has been reported that VEGF increases the expression of Bcl-2 in human umbilical vein endothelial cells, and the overexpression of Bcl-2 prevents the apoptotic cell death caused by growth factor deprivation in human umbilical vein endothelial cells.<sup>27</sup> Furthermore, the adenoviral gene transfer of Bcl-2 has been shown to reduce ischemia-reperfusion injury in the liver.<sup>28</sup> In the present study, it was demonstrated that Bcl-2 protein levels decreased dramatically after 8 hours of VEGF deprivation in isolated SECs (Fig. 7), which may be one of the key regulating factors of apoptosis under this condition. Surprisingly, glycine prevented this decrease of Bcl-2 protein levels almost completely. Although the precise mechanisms by which glycine prevented the decrease of Bcl-2 is unclear, these findings support the hypothesis that glycine prevents apoptosis of SECs by maintaining Bcl-2 protein levels.

In conclusion, glycine is protective against apoptotic cell death in SECs. Glycine most likely acts through glycine receptor on SECs, and it also prevents the decrease of Bcl-2 under VEGF deprivation. This antiapoptotic effect of glycine in SECs might be involved, at least in part, in the mechanism by which glycine prevents various types of liver injury in which apoptosis of SECs plays an important role.

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