Title

Glycine exerts dose-dependent biphasic effects on vascular development of zebrafish embryos

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Abstract

Glycine, a non-essential amino acid, is involved in both angiogenesis and anti-angiogenesis. We hypothesized that glycine would exert dose-dependent different effects on angiogenesis. In this study, we investigated the effects of a broad range of concentrations of glycine on vascular development using transgenic zebrafish *Tg(fli1a:Myr-mCherry)*^{ncv1} embryos. Effects of glycine transporter (GlyT) inhibitors (sarcosine and bitopertin) and a glycine receptor (GlyR) inhibitor (strychnine) were also examined in embryos in the absence or presence of glycine. After exposure to glycine and inhibitors, intersegmental vessels (ISVs) were observed by fluorescent microscopy. Low concentrations of glycine promoted the development of ISVs, whereas high concentrations reduced it. These effects of glycine could generally be reversed by treatment with GlyT and GlyR inhibitors. Furthermore, expressions of *vascular endothelial growth factor (VEGF)* (an angiogenic factor) and *nitric oxide synthase* (*NOS)* (an enzyme for nitric oxide synthesis) were associated with the dose-dependent effects of glycine. Our results suggest that glycine exerts dose-dependent biphasic effects on vascular development, which rely on GlyTs and GlyRs, and correlate with the expression of *VEGF* and *NOS* genes. At low concentrations, glycine acted as an angiogenic factor. In contrast, at high concentrations, glycine induced anti-angiogenesis. This evidence provides a novel insight into glycine as a unique target in angiogenic and anti-angiogenic therapy.

Keywords

Glycine; angiogenesis; zebrafish; VEGF; NOS

Introduction

Angiogenesis is the expansion of the vascular network from the existing vasculature into an avascular area [1]. Disruptions of various angiogenic signals, including VEGF, result in embryonic death at early stages [2], suggesting that angiogenesis is an essential process for development. In addition, angiogenesis accompanies pathological processes in cancer and chronic inflammatory diseases (e.g., psoriasis and rheumatoid arthritis) [3]. Therefore, it is critical to understand the role and functions of novel molecules, which regulate angiogenesis.

Glycine is a non-essential amino acid, which is mainly synthesized in the body from serine by the enzyme serine hydroxymethyltransferase [4]. GlyTs and GlyRs are present in various cell types, including endothelial cells (ECs) [5,6,7].

It has been established that glycine acts as a major inhibitory neurotransmitter in the central nervous

system [7,8]. The specific uptake of glycine is via high affinity for two GlyTs: GlyT1, which is mainly present in neighbouring glial cells; and GlyT2, which is mainly present in presynaptic nerve terminals [9]. GlyTs contribute to the control of the extracellular levels of glycine. Glycine is stored in and released from nerve terminals. In response to glycine, GlyRs, which is ligand-gated ion channels, allow an influx of Cl^- through the cell membrane, resulting in control of the excitability of the central nervous system [10].

VEGF is an essential regulator of angiogenesis that drives EC functions, including survival, proliferation, migration, and morphological changes [2,11,12,13]. In addition to its function in neurotransmission, glycine has also been reported to exert protective effects against apoptosis resulting from VEGF deprivation in rat ECs [14]. Intracellular glycine in human umbilical vein endothelial cells (HUVEC) was increased by VEGF [6]. Glycine promoted network formation of HUVEC and angiogenesis on the chicken chorioallantoic membrane [6]. These studies indicated that glycine could benefit therapeutic angiogenesis. On the other hand, the inhibitory effects of glycine have been reported in the context of tumour-angiogenesis. Dietary glycine decreased tumour growth and tumour-angiogenesis formed after implantation of cancer cell lines [5,15,16]. Although glycine unaffected tumour cell growth, it reduced cell proliferation of ECs [5,16]. These reports suggest that

glycine may participate in tumour suppression by preventing EC growth. Seemingly conflicting effects of glycine on angiogenesis have been reported, and the underlying role of glycine remains enigmatic. In the present study, we aimed to identify the role of glycine in angiogenesis using zebrafish embryo models.

Material and methods

Materials

Glycine was purchased from Sigma–Aldrich (Cat. No. G7126). Sarcosine/N-methylglycine (Cat. No. 30542-22; Nacalai Tesque), bitopertin (also known as RG1678 or RO4917838; Cat. No. 129-04861; Cayman Chemical, Ann Arbor, MI, USA), and strychnine (Cat. No. 32316-74; Nacalai Tesque) were used.

Zebrafish maintenance and embryo treatment

Experiments were approved and performed in accordance with the institutional guidelines established by the Committee on Animal Experimentation of Hokkaido University (approval no. 16-0084). Transgenic zebrafish *Tg(fli1a:Myr-mCherry)*^{ncv1} [17], which have been used for studying vascular

development [18], were obtained from the National BioResource Project. Adult zebrafish were maintained under standard conditions with a 14 h light/10 h dark cycle at 28 °C, as previously described [19]. Embryos were obtained by breeding and collected in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; pH 7.4). At 3 h post fertilization (hpf), embryos were randomly assigned, placed, and cultured in a 24-well plate (10 embryos per well) with 500 μL of E3 solution containing an indicated concentration of glycine (Fig. 1A). Inhibitors were applied at 9 hpf, and embryos were maintained until 28 or 30 hpf (Fig. 1B). Survival rates were manually calculated at 24 hpf. At the end of the treatment period, zebrafish embryos were dechorionated with 0.025% actinase E (Cat. No. 29003-64; Nacalai Tesque, Kyoto, Japan) in E3 solution. Following euthanasia using tricaine (Cat. No. E10521; Sigma−Aldrich, St. Louis, MO, USA) and cooling, embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C.

Analysis of vessel sprouting

Whole body images of zebrafish embryos were obtained using a Nikon ECLIPSE TS100 inverted microscope and Nikon DS-Fi3 Digital Camera (Nikon Corporation, Tokyo, Japan). Images were converted to 8-bit images, and brightness and contrast were uniformly adjusted across the entire image using ImageJ software version 1.44o (National Institutes of Health, Bethesda, MD, USA).

Images of vascular structure in the midtrunk region of all embryos, excluding those destroyed or not showing fluorescence, were acquired using Axio Imager 2 (Zeiss, Germany, Oberkochen) and IX71 (Olympus, Tokyo, Japan) fluorescence microscopes. Brightness and contrast were adjusted uniformly across the original images using ImageJ software for clarity of vessel structures. The number and length of ISVs were counted or measured in a blinded manner (Fig. 1A). The lengths of ISVs were measured (five vessels per one embryo) by manually tracing each vessel structure using ImageJ software. The total number of embryos (analysis of ISV number and survival rates) or vessels (analysis of ISV length) from three independent experiments is indicated in the brackets in each Figure.

Total RNA extraction and reverse transcription-PCR (RT-PCR) analysis

Total RNA was isolated from whole zebrafish embryos using QIAzol Lysis Reagent (Cat. No. 79306; Qiagen, Venlo, Netherlands). The RT reaction was performed with 300 ng of total RNA prepared from each sample using an Omniscript RT Kit (Ca. No. 205111; Qiagen) with a mixture of oligo(dT) and random primers. The RT product (1 μL) was subjected to PCR amplification (15 μL) using a Taq PCR Core Kit (Ca. No. 201223; Qiagen) in a standard thermal cycler PC707 (ASTEC, Fukuoka, Japan). Thermal cycling conditions were as follows: 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s.

The following primers were used: *vegfaa,* fwd 5'-CTCCTCCATCTGTCTGCTGTAAAG-3' and rev 5'-CTCTCTGAGCAAGGCTCACAG-3' (29 cycles, product size: 490 bp); *nos2a*, fwd 5'-GTGTTCCCTC AGAGAACAGAT-3' and rev 5'-GATCAGTCCTTTGAAGCTGAC-3' (35 cycles, product size: 822 bp); and *elfa,* fwd 5'-CTTCTCAGGCTGACTGTGC-3' and rev 5'-CCGCTAGCATTACCCTCC-3' (29 cycles, product size: 358 bp). PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with SYBR Gold Nucleic Acid Gel Stain (Cat. No. S11494; Molecular Probes, Eugene, OR, USA), followed by detection under a UV transilluminator (Red Imaging System; Alpha Innotech, San Diego, CA, USA). PCR bands were quantitated by densitometry using the ImageJ software in a blinded manner and normalized to the levels of *elfa*.

Statistical analysis

Experimental results are indicated as the mean ± SEM (analysis of vessel sprouting) or SD (analysis of gene expression). Statistical significance was determined by Dunnett's multiple comparison test using the MEPHAS webtool (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/dunnett-e.html). Differences with $p < 0.05$ were considered statistically significant.

Results

Glycine exerts biphasic effects on the vascular development of zebrafish embryos

We assessed vascular development in transgenic zebrafish, *Tg(fli1a:Myr-mCherry)*^{ncv1} embryos to investigate the role of exogenous glycine in angiogenesis *in vivo* (Fig. 1A). Embryos were maintained in E3 solution, and exposed to glycine with or without inhibitors. Following the fixation of embryos at the end of the treatment period, vascular features in the midtrunk region were observed and measured. At approximately 20 hpf, ISVs begin to sprout from the dorsal aorta, elongate and branch, eventually leading to the formation of the dorsal longitudinal anastomotic vessels (DLAVs). This primary network is fully formed by approximately 36 hpf [20,21]. Thus, we set up three schedules for analysis (Fig. 1B). Embryos were exposed to 0, 1, 10, 100, 200, 400, and 600 mM of glycine from 3 hpf, and observed at 28 hpf (Fig. 1B (a)). Embryos were treated with inhibitors at 5, 10, and 50 μM concentrations from 9 hpf, and observed at 30 hpf (Fig. 1B (b)). Embryos were exposed to glycine (10 and 400 mM) from 3 hpf, treated with inhibitors (10 μM) from 9 hpf, and observed at 28 and 30 hpf (Fig. 1B (c)).

Firstly, embryos were exposed to glycine at a broad range of concentrations (Fig. 1B (a), C and D, and Fig. 2). Embryos exposed to 0–400 mM glycine had a high survival rate at 24 hpf (Fig. 1C), and did not show differences in gross morphology (Fig. 1D). Exposure to 600 mM glycine caused death due to severe growth retardation (Fig. 1C and D). The number of ISVs was unaffected in embryos exposed to 0–400 mM glycine (Fig. 2A and B). The length of ISVs sprouting in embryos treated with 10 and 100 mM glycine, but not 1 mM glycine, was increased and reached DLAV position, compared with the partial sprouting observed in embryos exposed to 0 mM glycine (Fig. 2A and C). These results suggested that these low concentrations of glycine promote vascular sprouting. There was no significant difference in the length of ISVs between 200 mM glycine and controls. However, exposure to 400 mM glycine decreased the length of ISVs, indicating the inhibition of angiogenesis. Taken together, these findings propose that glycine may exert biphasic dose-dependent effects on vascular development.

VEGF and nitric oxide (NO) are important factors for angiogenesis [22,23]. We evaluated the expression of *vegfaa* and *nos2a* genes in embryos exposed to glycine for 3–30 hpf. Consistent with the angiogenic responses to glycine, exposure to 10 mM glycine upregulated the expression of *vegfaa* and *nos2a* genes (Fig. 2D). Although the exposure to 400 mM glycine unaffected the expression of

vegfaa, it markedly downregulated that of *nos2a* (Fig. 2D).

Effects of glycine on vascular development are blocked by inhibition of GlyTs and GlyRs

We assessed the contribution of GlyTs and GlyRs to vascular development. Embryos were treated with 5, 10, and 50 μM GlyT inhibitors (sarcosine and bitopertin), as well as a GlyR inhibitor (strychnine) at 9 hpf, and observed at 30 hpf (Fig. 1B (b)). At these concentrations, treatment with any of the inhibitors decreased the number and the sprouting length of ISVs (Fig. 3A and B). The survival rate under these conditions was unaffected (Fig. 3C). These findings suggest the role of endogenous glycine in vascular development acts through GlyTs and GlyRs.

We examined whether GlyTs and GlyRs are involved in the vascular development induced by a low concentration of glycine (10 mM). Embryos at 3 hpf were exposed to 10 mM glycine and, at 9 hpf, treated with sarcosine, bitopertin, or strychnine at a concentration of 10 μM (Fig. 1B (c)). There was no difference in the number of ISVs among 0 mM glycine groups and 10 mM glycine groups with or without these inhibitors (Fig. 4A). However, treatment with all these inhibitors prevented the increase in ISV length induced by 10 mM glycine alone (Fig. 4B). Notably, the survival rates remained unaffected (Fig. 4C). The expressions of *vegfaa* and *nos2a* genes were evaluated in embryos exposed

to glycine for 3–30 hpf. Addition of 10 μM bitopertin or strychnine prevented the increased expression of *vegfaa* and *nos2a* genes induced by 10 mM glycine (Fig. 2D and 4D).

Next, we assessed the influence of sarcosine, bitopertin, and strychnine on the inhibitory effects occurred with a high concentration of glycine (400 mM). Sarcosine and bitopertin at a concentration of 10 μM unaffected the number of ISVs and improved the decreased ISV length caused by 400 mM glycine; however, the observed recoveries were less extensive than the ISV lengths reported for 0 mM glycine controls (Fig. 4E and F). Notably, strychnine failed to attenuate the inhibitory effect of 400 mM glycine, and further decreased the ISV length. High survival rates were maintained in embryos treated with the combination of 400 mM glycine and inhibitors (Fig. 4G). The decreased expression of *nos2a* induced by 400 mM glycine was improved by bitopertin (Fig. 2D and 4H). Of note, strychnine further reduced the expression of the *vegfaa* gene in 400 mM glycine. These findings indicate that a low concentration of glycine may promote vascular sprouting, dependent on GlyTs and GlyRs. Interestingly, GlyTs appear to be required for the anti-angiogenic effects of a high concentration of glycine. Furthermore, VEGF and NOS may partially participate in the dose-dependent angiogenic response to stimulation by glycine at low and high concentrations.

Discussion

To the best of our knowledge, this is the first investigation on the role of glycine at a broad range of concentrations in the vascular development of zebrafish embryos. We found a biphasic effect of glycine on angiogenesis *in vivo*. Low concentrations (10 and 100 mM) of glycine facilitated vascular sprout elongation, and this increase was blocked by treatment with GlyT and GlyR inhibitors. In contrast, a high concentration (400 mM) of glycine obstructed vascular development, and this decrease was improved by GlyT inhibitors. These angiogenic and anti-angiogenic effects of glycine may be partially modulated by regulating the expression of *VEGF* and *NOS* genes.

Glycine has been reported to suppress tumour-angiogenesis [5,15,24], interestingly, also promote angiogenesis [6,14]. The seemingly inconsistent results in these studies on glycine may be related to differences in the species, tissues, and cells used, or may be attributed to different approaches (e.g., culture conditions, doses, and periods of time). A low concentration of glycine for a short period of time (140 μM, 5 h) promoted vessel network formation of HUVEC [6]. A high concentration of glycine for a long period of time (0.1–10 mM, 4 days) inhibited the proliferation of cow ECs [16]. In this study, we demonstrated the dose-dependent biphasic effect of exogenous glycine on angiogenesis *in vivo* (Fig. 2). Low-concentration glycine increased angiogenesis, whereas high-concentration

glycine caused anti-angiogenic effects. Our findings may partially explain the discrepancies in previous research.

It has been reported that endogenous glycine contributes to angiogenesis. Knockdown of *MTHFD2*, an enzyme for serine-glycine metabolism, decreased intracellular glycine levels in ECs and inhibited angiogenic functions; these effects were reversed by the supplementation of glycine [25]. Inhibition of GlyRs by strychnine attenuated the inflammatory angiogenesis in mice [26]. In line with previous reports, our data showed that monotherapy with sarcosine, bitopertin, and strychnine impaired zebrafish vascular development (Fig. 3). Furthermore, the biphasic effect of exogenous glycine also appears to partially depend on GlyTs and GlyRs (Fig. 4). In addition, the expression of the *VEGF* and *NOS* genes, crucial factors regulating various EC functions [12,13,22,27], partially correlated with the angiogenic and anti-angiogenic effects of glycine, and the reversal effects of GlyT or GlyR inhibitors on these glycine effects (Fig. 2D, and Fig. 4D and H).

Proteins present in GlyT and GlyR have been detected in HUVEC [5,6]. VEGF receptor *Kdrl*-expressing zebrafish ECs showed high expression of *GlyT1*, and moderate expression of *GlyT2* and *GlyR* subunits using RNA sequencing data (Gene Expression Omnibus accession: GSE119718).

Therefore, glycine may modulate angiogenesis through effects on EC functions. On the other hand, GlyT and GlyR genes have also been detected in non-ECs (GSE119718). Thus, future studies are warranted to address the detailed mechanisms of angiogenesis by glycine.

In this study, we exposed zebrafish embryos to a broad range of concentrations of glycine to investigate the role of glycine in angiogenesis. Our findings provide evidence of the concentration-dependent biphasic effect of exogenous glycine, which positively and negatively regulates angiogenesis *in vivo*, mainly relies on GlyTs and GlyRs. GlyT inhibitors, including bitopertin, are expected to be used in the treatment of mental disorders, such as chronic pain and schizophrenia [28,29]. Our study indicates that bitopertin may be able to normalize angiogenesis that is altered in the presence of low or high concentrations of glycine. These findings suggest that glycine signalling is a novel target for angiogenic and anti-angiogenic therapy.

Author contributions

K.T-T designed the research plan and performed experiments. M.S, M.F and M.T contributed to experiments and data analysis. All authors prepared and reviewed the manuscript.

Declaration of competing interest

The authors have no competing interests.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (KAKENHI 18K0978208). We thank to T. Simizu and N. Fujita (Graduate School of Dental Medicine, Hokkaido Univ.) for data analysis and fish maintenance.

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Figure legends

Fig. 1. The effects of glycine on the development of zebrafish

(A and B) Experimental design to assess the effects of glycine on the vascular development of zebrafish. (A) Embryos from zebrafish *Tg(fli1a:Myr-mCherry)*^{ncv1} were cultured in a 24-well plate (10 embryos per well), and exposed to glycine with or without inhibitors. At the end of the treatment period, embryos were dechorionated, euthanized, and fixed. The vascular structure in the midtrunk region was observed under a fluorescence microscope. DA, dorsal aorta; ISVs, intersegmental vessels; DLAVs, dorsal longitudinal anastomotic vessels. (B) Schedules of exposure to glycine with or without inhibitors. (a) Embryos were exposed to glycine (0, 1, 10, 100, 200, 400, and 600 mM) from 3 h post fertilization (hpf), and observed at 28 hpf. (b) Embryos were treated with inhibitors (5, 10, and 50 μM) from 9 hpf, and observed at 30 hpf. Control embryos were treated with 0.1% DMSO. (c) Embryos were exposed to glycine (10 and 400 mM) from 3 hpf, treated with inhibitors (10 μM)

from 9 hpf, and observed at 28 and 30 hpf. Survival rate was determined at 24 hpf. (C and D) At 28 hpf, embryos exposed to glycine (0–400 mM) showed normal gross morphology in the whole body; however, glycine (600 mM) caused severe developmental retardation and death. (C) The survival of embryos at 24 hpf. (D) Images of whole zebrafish embryos at 28 hpf. Scale bars indicate 200 μm. Similar results were obtained in from three independent experiments.

Fig. 2. Glycine exerts biphasic effects on the vascular development of zebrafish embryos

Glycine (10 and 100 mM) promoted the development of ISVs, whereas glycine (400 mM) had an opposite effect. (A) Fluorescent images of ISVs at 28 hpf. Scale bars indicate 100 μm. Arrows indicate ISVs. Arrowheads indicate the forming DLAVs. Similar results were obtained from three independent experiments. (B and C) The number (B) and length (C) of ISVs at 28 hpf. * Significantly different from control (glycine 0 mM) with $p < 0.05$. N/A, not applicable. (D) The expression of *vegfaa* and *nos2a* genes in embryos exposed to glycine (0, 10 and 400 mM) for 3–30 hpf was semi-quantified using RT-PCR. (Upper portion) Agarose gel images of PCR products of five samples obtained from three independent experiments. *elfa* was used as an internal control. (Lower portion) Expression levels were normalized to those of *elfa*. Data are presented as a ratio relative to a glycine (0 mM) group. * Significantly different from each control group with $p < 0.05$.

Fig. 3. Inhibition of glycine transporters (GlyTs) and receptors (GlyRs) impairs vascular development

GlyT inhibitors (sarcosine and bitopertin) and a GlyR inhibitor (strychnine) suppressed the development of ISVs at 5, 10, and 50 μM concentrations. (A and B) The number (A) and length (B) of ISVs at 30 hpf. * Significantly different from DMSO control with $p < 0.05$. (C) The survival of embryos at 24 hpf.

Fig. 4. Effects of glycine are reversed by inhibition of GlyTs and GlyRs

(A, B, C and D) The angiogenic effect of low glycine was blocked by inhibition of GlyTs and GlyRs. The increase in ISV development upon exposure to glycine (10 mM) was prevented by sarcosine, bitopertin, and strychnine (10 μM). (E, F, G and H) The anti-angiogenic effect of high glycine was attenuated by inhibition of GlyTs. The decrease in ISV development upon exposure to glycine (400 mM) was prevented by sarcosine and bitopertin (10 μM). (A and E) The number of ISVs at 28 hpf (A) or 30 hpf (E). * Significantly different from a glycine (0 mM)-DMSO group with $p < 0.05$. (B and F) The length of ISVs at 28 hpf (B) or 30 hpf (F). * Significantly different from a glycine (0 mM)-DMSO group with $p < 0.05$. (C and G) The survival of embryos at 24 hpf. (D and H) The

expression of *vegfaa* and *nos2a* genes in embryos exposed to glycine (for 3-30 hpf) with inhibitors (for 9-30 hpf) was semi-quantified using RT-PCR. (D) Glycine (10 mM) with added bitopertin or strychnine (10 μM). (H) Glycine (400 mM) with added bitopertin or strychnine (10 μM). (Upper portion) Agarose gel images of PCR products of five samples obtained from three independent experiments. *elfa* was used as an internal control. (Lower portion) Expression levels were normalized to those of *elfa*. Data are presented as a ratio relative to each control group: a glycine (10 mM)-DMSO group (D) or a glycine (400 mM)-DMSO group (H). * Significantly different from each control group with $p < 0.05$.

Figure 1

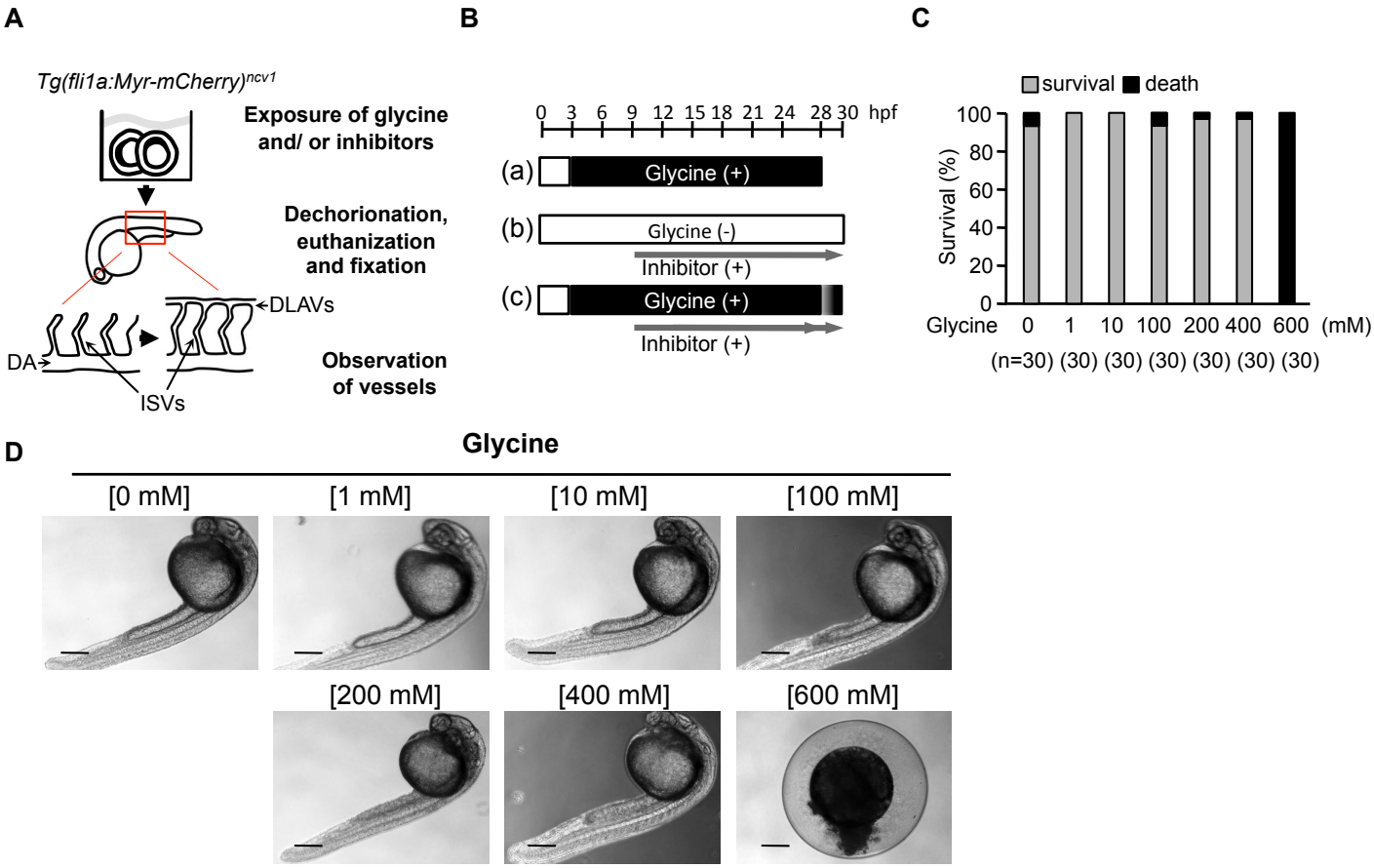

A
Tg(fli1a:Myr-mCherry)ncv1
Exposure of glycine and/ or inhibitors
Dechorionation, euthanization and fixation
DLAVs
DA
ISVs
Observation of vessels
B
0 3 6 9 12 15 18 21 24 28 30 hpf
(a) Glycine (+)
(b) Glycine (-)
Inhibitor (+)
(c) Glycine (+)
Inhibitor (+)
C
survival death
Survival (%)
100 80 60 40 20 0
Glycine 0 1 10 100 200 400 600 (mM)
(n=30) (30) (30) (30) (30) (30) (30)
D
Glycine
[0 mM]
[1 mM]
[10 mM]
[100 mM]
[200 mM]
[400 mM]
[600 mM]

Figure 2

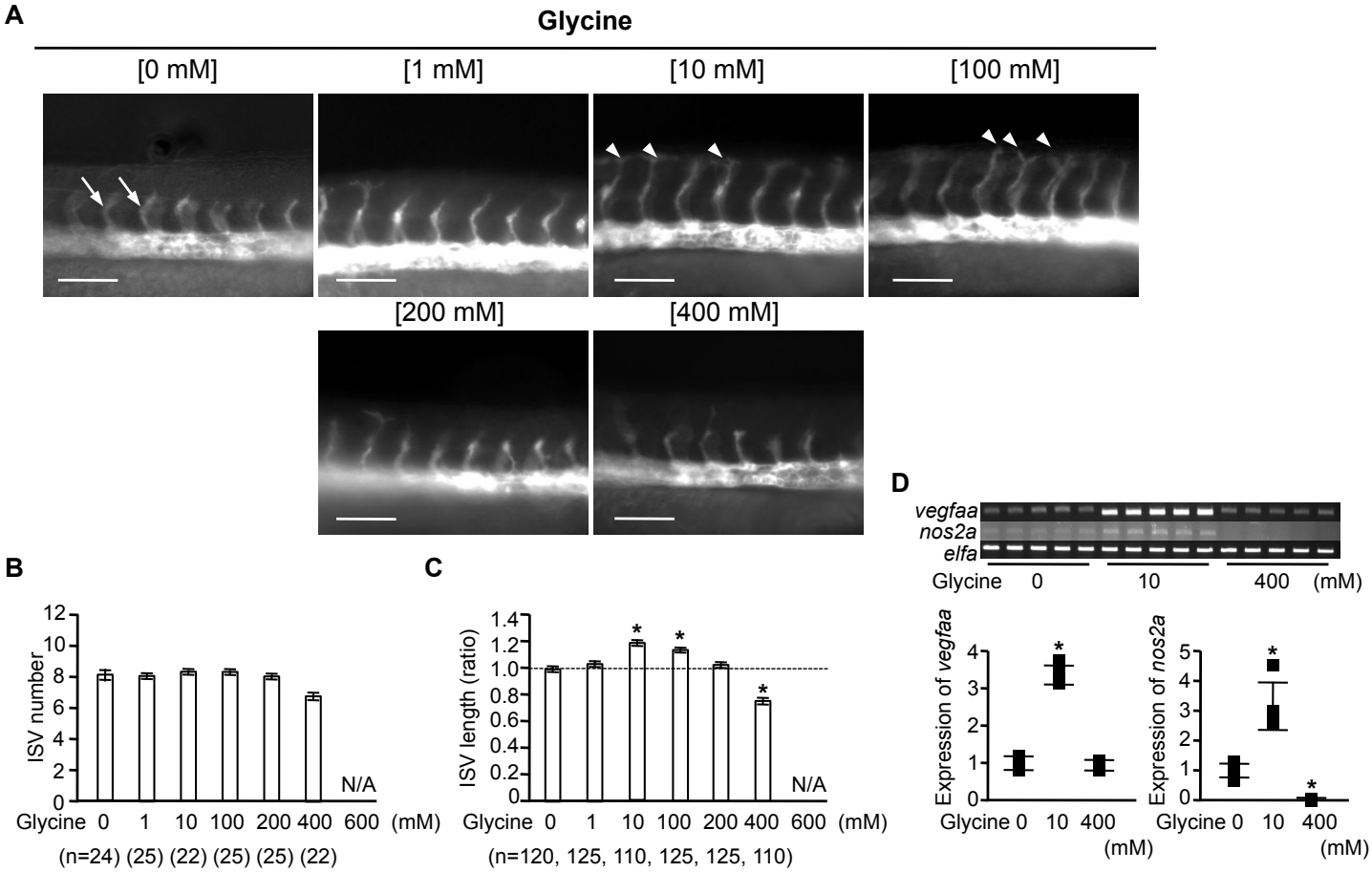
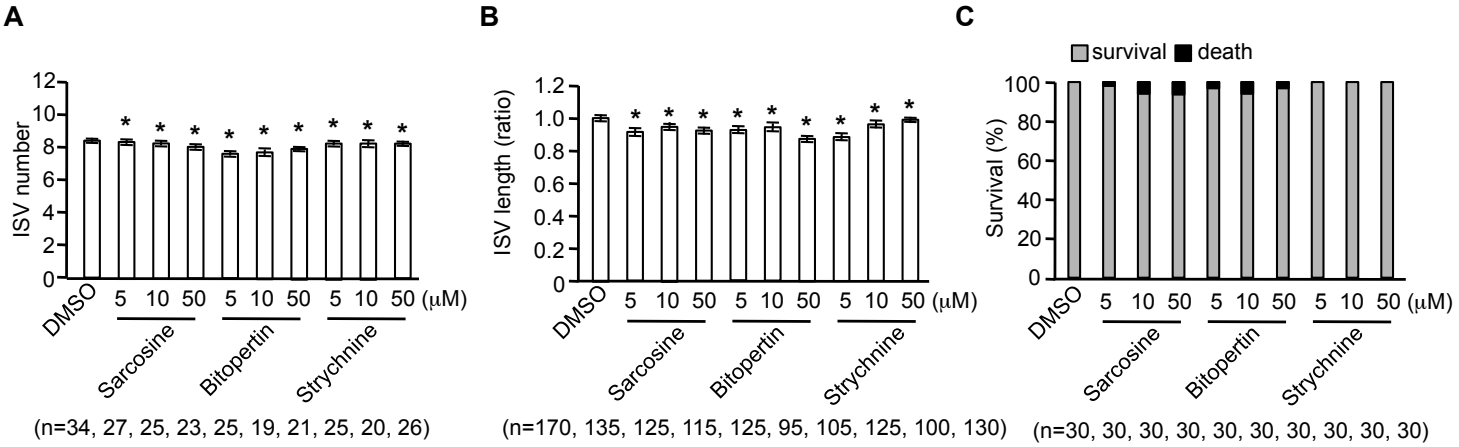

A
Glycine
[0 mM]
[1 mM]
[10 mM]
[100 mM]
[200 mM]
[400 mM]
B
ISV number
N/A
Glycine 0 1 10 100 200 400 600 (mM)
(n=24) (25) (22) (25) (25) (22)
C
ISV length (ratio)
N/A
Glycine 0 1 10 100 200 400 600 (mM)
(n=120, 125, 110, 125, 125, 110)
D
vegfaa
nos2a
elfa
Glycine 0 10 400 (mM)
Expression of vegfaa
Glycine 0 10 400 (mM)
Expression of nos2a
Glycine 0 10 400 (mM)

Figure 3

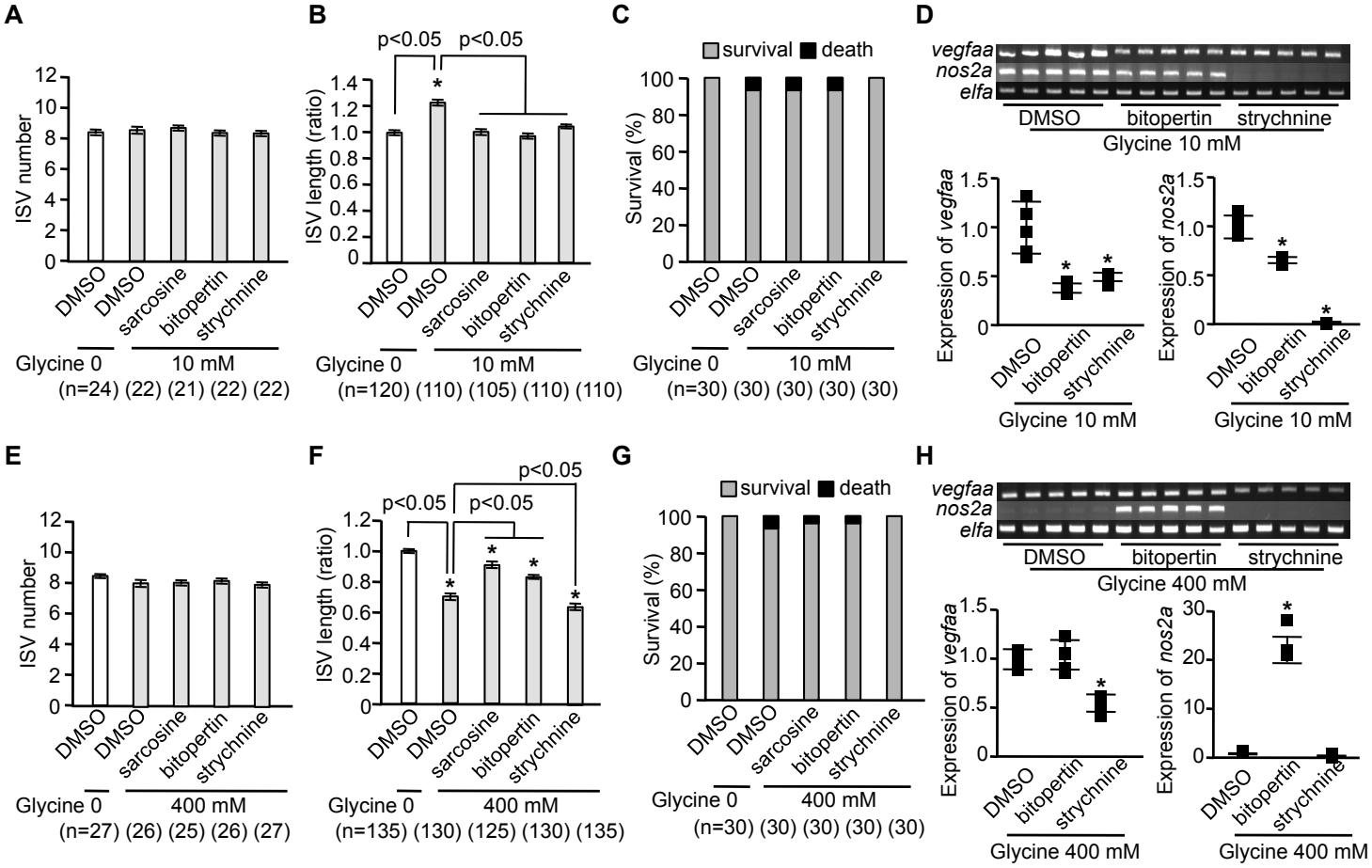


(n=34, 27, 25, 23, 25, 19, 21, 25, 20, 26)

(n=170, 135, 125, 115, 125, 95, 105, 125, 100, 130)

(n=30, 30, 30, 30, 30, 30, 30, 30, 30, 30)

Figure 4


A
ISV number
DMSO
DMSO
sarcosine
bitopertin
strychnine
Glycine 0
10 mM
(n=24) (22) (21) (22) (22)
B
p<0.05
p<0.05
ISV length (ratio)
Glycine 0
10 mM
(n=120) (110) (105) (110) (110)
C
survival
death
Survival (%)
Glycine 0
10 mM
(n=30) (30) (30) (30) (30)
D
vegfaa
nos2a
elfa
DMSO
bitopertin
strychnine
Glycine 10 mM
Expression of vegfaa
Expression of nos2a
Glycine 10 mM
Glycine 10 mM
E
ISV number
Glycine 0
400 mM
(n=27) (26) (25) (26) (27)
F
p<0.05
p<0.05
p<0.05
ISV length (ratio)
Glycine 0
400 mM
(n=135) (130) (125) (130) (135)
G
survival
death
Survival (%)
Glycine 0
400 mM
(n=30) (30) (30) (30) (30)
H
vegfaa
nos2a
elfa
DMSO
bitopertin
strychnine
Glycine 400 mM
Expression of vegfaa
Expression of nos2a
Glycine 400 mM
Glycine 400 mM