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Therapeutic angiogenesis of mouse hind limb ischemia by novel peptide activating GRP78 receptor on endothelial cells

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ABSTRACT

Therapeutic angiogenesis emerged as a non-invasive mean of promoting neovascularization in ischemic tissues. We have searched for new molecules that induce angiogenesis by screening a phage display combinatory peptide library on endothelial cells. One of the selected peptides identified by binding to endothelial cells under hypoxic conditions was further studied. The aim of this study was to assess the therapeutic value of this peptide, RoY, in a mouse hind limb ischemia model and to identify it's receptor on endothelial cells.

RoY, a 12 amino-acid synthetic peptide, induced *in vitro* angiogeneic activity under hypoxic conditions by increasing endothelial cell proliferation, migration and tube formation. In order to assess its therapeutic properties in ischemic tissues, a hind limb ischemia model was induced in C57BL mice by a femoral artery excision. A single local intramuscular injection of RoY peptide to the operated limb, significantly restored blood perfusion and alleviated hind limb ischemia as determined by a laser Doppler imager. Increased capillary density in histological sections corroborated these findings. Protein precipitation and mass spectroscopy studies identified GRP78, a heat shock protein, as the peptide-binding membrane receptor that was increased on endothelial cell membranes under hypoxic conditions. This study demonstrates the efficacy of RoY peptide in alleviation of hind limb ischemia. In addition, it provides evidence that GRP78 is an angiogenic receptor on hypoxic endothelial cells.

1. Introduction

Angiogenesis is the natural process of new blood vessel formation by the sprouting of endothelial cells from pre-existing vessels [1]. Given the adverse implications of a hypoxic environment (oxygen deficiency in tissues) on physiological and pathological processes, in addition to the current epidemic of ischemic diseases, researchers are seeking innovative treatments [2]. Therapeutic angiogenesis has emerged as a non-invasive mean of promoting revascularization in underperfused tissues in order to improve the local hypovascularity in myocardial infarction, stroke and peripheral artery diseases [3–5]. Therefore, recent experimental and clinical studies have focused on the efficacy of interventions with growth factors, with known angiogenic properties. Vascular endothelial growth factor (VEGF) is one of the most specific regulators of angiogenesis in endothelial cells that has been found to elicit pronounced angiogenic responses in a wide variety of *in vivo* models. However, clinical studies of various modes of angiogenic therapies using VEGF

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have yielded disappointing results [6–9]. As many aspects of angiogenesis are still poorly understood, the challenge lies in identifying pathways that might require a novel approach to angiogenesis induction.

In an earlier study, we screened a phage display combinatory peptide library on human umbilical endothelial cells. We identified a group of novel peptides that bound to endothelial cells under different physiological conditions and induced angiogenesis *in vitro*. Injection of selected peptides into mouse ears led to an increase in the number of blood vessels. These synthetic peptides did not induce VEGF receptor gene expression, suggesting a possible VEGF-independent mechanism [10]. In the present study we evaluated the angiogenic activity under hypoxic conditions of the peptide selected by screening endothelial cells under hypoxic conditions. RoY peptide was assessed for its therapeutic value in alleviating hind limb ischemia. Furthermore, in this study we identified its membrane-binding receptor on endothelial cells.

2. Materials and methods

2.1. Endothelial cell culture

Endothelial cells were isolated from human umbilical veins by collagenase digestion [11]. This study conforms the principles outlined in the Declaration of Helsinki for use of human tissues. Endothelial cells were cultured in 30 mm fibronectin-covered Petri dishes with M199 supplemented with 20% fetal calf serum (FCS), 10,000 units penicillin, 10 mg/ml streptomycin sulfate, 10 mg/ml neomycin sulfate (Biological Industries, Kibbutz Beit Haemek, Israel), 25 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA, USA) and 5 U/ml heparin (Sigma, Rehovot, Israel). The isolated endothelial cells were trypsinized and expanded in 25 cm^2 tissue culture flasks containing supplemented endothelial cell growth medium (PromoCell, Heilderberg, Germany), followed by incubation with 5% CO_2 at 37 °C. Cells from passages 3 to 4 were used for the different experiments. Each *in vitro* assay was repeated using three to six different cords. Cultures were subjected to hypoxic conditions using a gas mix of 94% nitrogen + 5% CO_2 + 1% O_2 in a hypoxia chamber (Billups-Rothenberg, San Diego, CA, USA).

2.2. Peptides synthesis

Peptides RoY and its scrambled form, sRoY, were synthesized by SynPepCA, USA. HPLC purity was more than 97% for each peptide synthesized. Peptides were disolved in water and diluted to a concentration of 1 mg/ml. The sequences of the RoY and scrambled RoY (sRoY) peptide are presented in Table 1.

Table 1

Peptide	Sequence
RoY	Y P H I D S L G H W R R
Scrambled RoY	R Y H L I P R G W D H S

2.3. Peptide binding to endothelial cells (FACS analysis)

Biotinylated RoY peptide was used for staining endothelial cells (6 μg per 100,000 cells) for 2 h on ice under normoxic conditions, or for 1.5 and 5 h under hypoxic conditions. The cells were washed twice with PBS, and streptavidin-fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, Stanford, CA, USA) was added for additional 30 min on ice. Streptavidin-FITC was used as the isotype control. FACS analysis was performed on gated live endothelial cells. Samples were analyzed using a fluorescence-activated cell sorter (FACScan, Beckton Dickinson, Franklin Lakes, NJ, USA). The experiments were repeated three times using endothelial cells from different cords in each experiment.

2.4. Endothelial cell proliferation

Proliferation of endothelial cells was measured by [H^3]thymidine incorporation. Cells seeded on 24-well plates (12,000 cells/well) were first cultured in endothelial cell growth medium for 24 h, that was replaced by supplement-free medium containing only 5% FCS. RoY peptide or its scrambled form peptide sRoY, was added at 0.1, 1, 10 and 100 ng/ml in triplicates for either 72 h under normoxic conditions or 24 h under hypoxic conditions followed by 48 h under normoxic conditions. The cells were pulsed with [H^3]thymidine (2 μCi/well) (Sigma, Rehovot, Israel) overnight, lysed by 15 min incubation at 37 °C with 300 μl/well of 0.5M NaOH and counted in a beta counter. Summary of the results obtained from four repeated experiments were expressed as the percent increase in thymidine incorporation in samples with peptides over samples without peptides ± S.E.M.

2.5. Endothelial cell migration

Endothelial cell migration was evaluated with the Chemicon QCM 96-well Migration Assay kit (Chemicon International, Temecula, CA, USA). A total of 25,000 endothelial cells per well were placed on the bottom of an 8 μm pore-size membrane in supplement-free medium. RoY peptide and sRoY peptide were added to the feeder tray at 5, 10, 20 and 50 ng/ml for 5 h under normoxic or hypoxic conditions. Cells that migrated through the membrane were lysed by the addition of a buffer containing CyQuant GR dye (Molecular Probes, Eugene, OR, USA) and fluorescence was measured with an ELISA reader at 480/520 nm. Results were expressed as net (over control – without peptide) relative fluorescence units. We repeated four times the migration assay using endothelial cell originating from different cords.

2.6. Tube formation

Endothelial cells were incubated in starvation conditions (medium without supplements of serum and growth factors) for 24 h in either hypoxic or normoxic conditions. Samples of 50,000 cells in 500 μl medium were transferred to 24-well plates precoated with 250 μl Cultrex Basement Membrane Extract (with reduced growth factors, R&D Systems, Minneapolis, MN, USA). RoY peptide was added at one optimal concentration of 10 ng/ml (based on preliminary findings). In

addition, sRoY was added at the same concentration and the slides were examined by light microscopy after 18 h incubation. The length of the network of connected cells (tube formation) was measured in micrometers in five different areas of each well using Image-Pro Plus Image software (Media Cybernetics, Silver Spring, MD, USA). Experiments were repeated three times using three different cords.

2.7. Ischemic hind limb model

Female C57BL6/J mice, aged 13–15 weeks, were used to evaluate the peptide's angiogenic activity [12–14]. This study was conducted with permission of the Institutional Animal Care and Use Committee of Tel Aviv University conforming to the NIH Guide for the Care and Use of Laboratory Animals. The mice were anesthetized by intraperitoneal injection of ketamine 80 mg/kg and xylazine 16 mg/kg, and a longitudinal incision was made on the medial side of the right thigh, parallel to the proximal artery–vein–nerve complex. The external iliac artery was isolated from the vein and nerve, ligated twice and dissected between the two ligatures. One day after surgery, mice were divided into four groups ($n = 16$) as follows: groups 1 and 2 were injected with 0.1 and 1 μg/mouse RoY peptide. Groups 3 and 4 (controls) were injected with PBS and scrambled RoY (1 μg/mouse). All injections were made intramuscularly at a site close to the ligation. Mouse limbs were scanned with a Laser Doppler Imager (Perimed, Jarfalla, Sweden) under general anesthesia, at room temperature (25 °C) immediately after surgery and on postoperative days 7, 14 and 21. To analyze the results, we gated the entire limb, including the foot. The median perfusion of each limb was determined, and the ratio between the ischemic (right) and the control, non-operated (left) limb (relative perfusion) was calculated.

2.8. Immunohistochemistry

On days 7 and 14, three animals in each group were euthanized, and each leg was cut into thigh and calf for preparation of paraffin blocks for histological analysis, as previously described [15,16]. On day 21, the remaining animals were sacrificed, and the excised limbs were immediately fixed with 4% formalin for 48 h and then embedded in paraffin. Each block was cut transversely into 3 μm thick sections in sequential order, and samples of the gastrocnemic muscle sections were taken from the same areas from the control and treated mice. Sections obtained from the calf were stained with rabbit anti-mouse von Willebrand factor (Dako Cytomation, Glostrup, Denmark) (primary antibody), and the capillaries were visualized with the Envision + System-Horseradish Peroxidase (HRP) kit (Dako Cytomation). Capillaries were counted under a microscope in a total of 10 random fields from different sections. Density was expressed as the mean number of capillaries per field of view.

2.9. Identification of RoY peptide-binding protein

Endothelial cells cultured in 90 mm Petri dishes for 24 h in complete Endothelial Cell Growth Medium (PromoCell, Heildelberg, Germany) were exposed to normoxic or hypoxic conditions and lysed after 20 min on ice with buffer lysate (50 mM TrisCl, pH 8, 150 mM NaCl, 0.02% Na azide, 0.1% sodium deodocyl sulfate, 1% NP-40, 1 μg/ml protease inhibitors, 100 μg/ml phenylmethylsulphonylfluoride). The lysates were centrifuged (10,000 rpm) and supernatant samples (50 μg/500 μl) were precleared with agarose–streptavidin–biotin and then precipitated with 10 μg of biotinylated RoY peptide for 1 h at 4 °C. Streptavidin Sepharose beads (Amersham Biosciences, Uppsala, Sweden) (50 μl) were then added to the samples for 1 h at 4 °C. Samples were centrifuged at 12,000 × g for 20 s, and the pellet was subjected to polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie blue, and the major stained band was cut from the gel and examined by mass spectroscopy for identification of its protein content. The procedure for protein identification included digestion of the cut gel band by trypsin and analysis by LC–MS/MS on LTQ-Orbitrap (Thermo) and identification by Pep-Miner and Sequest software against the human, mouse, rat, bovine and rabbit part of the database.

2.10. Verification of RoY-binding protein by Western blot analysis

Precipitated endothelial cells lysates as described above, were analyzed by Western blot in order to verify that the protein precipitated binds RoY peptide. Nitrocellulose membranes were stained with either biotinylated RoY (5 μg/ml in PBS–Tween) or anti-GRP78 polyclonal antibody (C-20, sc-1051, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C and incubated with 1 μg/ml anti-HRP-conjugated Streptavidin (Jackson ImmunoResearch) for the biotinylated peptide or anti-goat HRP (Jackson ImmunoResearch) for the GRP78 protein, followed by SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

2.11. FACS analysis of RoY peptide binding to GRP78 receptor on endothelial cells

Endothelial cells were harvested by trypsin and 100,000 cells per sample were suspended in PBS containing 5%FCS and 0.1% Na azide. RoY and sRoY peptides were added at increasing concentrations to endothelial cells for 1 h on ice. Goat polyclonal anti-GRP78 (Santa Cruz, Biotechnologies, CA, USA. Ca. No. N-20 sc-1050) in a concentration of 1 μg/100,000 cells was added for an additional 1 h. Cells were washed and stained with anti-goat FITC (Jackson ImmunoResearch Laboratories, PA, USA). Samples were analyzed using a fluorescence activated cell sorter (FACScan Beckton Dickinson, CA, USA).

2.12. Anti-GRP78 binding to endothelial cells

Endothelial cells from 10 samples of human cord blood were cultured in endothelial cell growth medium for 24 h under normoxic conditions or 5 h under hypoxic conditions. The cells were removed and stained with anti-GRP78 polyclonal antibody (Santa Cruz, Biotechnologies. Ca. No. N-20 sc-1050) 2 μg/100,000 cells for 2 h on ice. Anti-goat FITC (Jackson ImmunoResearch) added for 30 min on ice. IgG1-FITC was used as the isotype control. FACS analysis was performed on

gated live endothelial cells to determine surface GRP78 and to avoid binding to internal GRP78 in dead cells. The samples were analyzed with a FACScan (Beckton Dickinson).

2.13. Statistical analysis

Analysis of variance and the Tukey–Kramer honestly significant difference (HSD) test for multiple comparisons were used for the statistical analysis. Results were considered significant at $p < 0.05$. JMP software (SAS Institute, Cary, NC, USA) was used for data management and analyses.

3. Results

3.1. RoY peptide binding to endothelial cells under hypoxic conditions

Binding of biotinylated RoY peptide to endothelial cells under normoxic and hypoxic conditions was studied by FACS analysis. FACS analysis was performed on gated live endothelial cells using one concentration of the peptide (6 μg per 100,000 cells, determined as optimal concentration in previous experiments). An increased number of peptide-binding cells was analyzed after 1.5 and 5 h under hypoxia compared to binding under normoxic conditions (Fig. 1A). Fig. 1B summarizes three repeated experiments and demonstrates that RoY peptide binds a significant ($p < 0.05$) higher percent (88 ± 2.8) of endothelial cells after 5 h of hypoxia compared to $56.5 \pm 0.7\%$ binding after 1.5 h of hypoxia and $16 \pm 1.1\%$ under normoxic conditions.

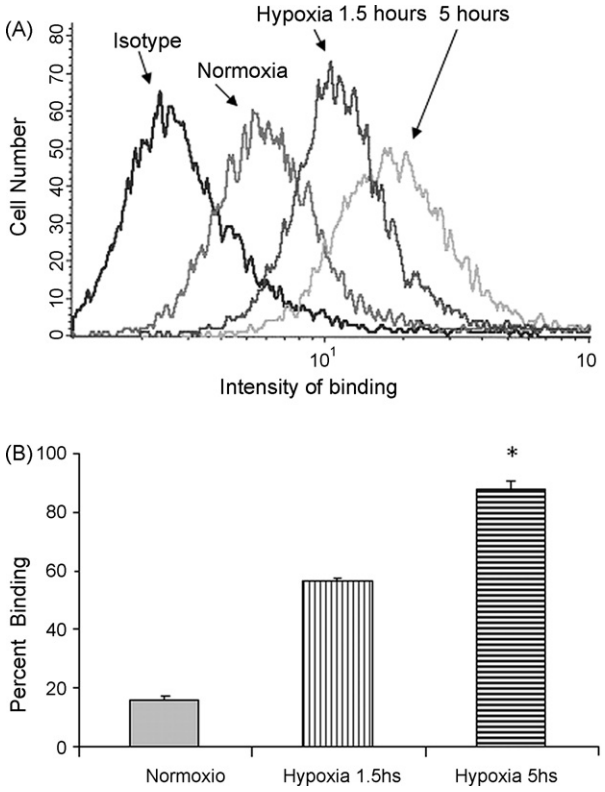

Fig. 1 – FACS analysis of biotinylated RoY peptide binding to endothelial cells under hypoxic and normoxic conditions. The FACS analysis (A) demonstrates the increasing binding of the RoY peptide to endothelial cells under hypoxia and (B) summarizes three repeated experiments demonstrating significant increase ($^*p < 0.05$) in percent of RoY-binding endothelial cells after 5 h of hypoxia. Values represent means ± S.D. of percent binding.

3.2. In vitro angiogenic effects of RoY peptide on endothelial cells

In order to evaluate the *in vitro* angiogenic properties of RoY peptide under hypoxic conditions, three well-established angiogenic parameters were studied:

A. *Cell proliferation* Endothelial cell proliferation in response to increasing peptide concentrations was measured by [H^3]thymidine incorporation in supplement-free medium. As can be seen in Fig. 2A, percent thymidine incorporation in cells with RoY peptide over cells without peptide was increased in both normoxic and hypoxic conditions to 124% at 0.1 ng/ml and 178% at 1 ng/ml. Under hypoxia only, RoY peptide at 10 ng/ml induced further thymidine uptake that reached maximum average of 207% ($p < 0.05$). However, at 100 ng/ml proliferation of hypoxic cells decreased to an average of 154% over control, while normoxic cells maintained control values. Proliferation was repeated four times using cell from different cords. Scrambled peptide (sRoY) did not induce significant proliferation under either normoxic or hypoxic conditions.
B. *Cell migration* determined the relative number of endothelial cells migrated above control (control being the number of migratory cells without the addition of peptides) to the chemoattractant peptides. The migratory chambers were incubated under either hypoxic or normoxic conditions and RoY and sRoY peptides in increasing concentrations were used as chemoattractants. As can be seen in Fig. 2B, significant migration was induced by a concentration of 20 and 50 ng/ml RoY in hypoxic condition and at 50 ng/ml RoY under normoxia ($p < 0.0001$). Five and 10 ng/ml of RoY peptide did not induce significant migration as well as sRoY control peptide.
C. *Tube formation* determined the ability of endothelial cells to form network of connected cells in tube-like structures in response to angiogenic stimulation. As can be seen in Fig. 2C, the network of connected cells was significantly longer for endothelial cells incubated with RoY peptide than for cells incubated with sRoY or untreated cells, under both normoxic and hypoxic conditions ($p < 0.005$). Illustrations of the cell cultures appear in Fig. 2D.

3.3. In vivo therapeutic effect of RoY peptide

In order to assess the therapeutic effect of RoY peptide in ischemic diseases, we used the mouse hind limb ischemia model, previously described for evaluation of angiogenic factors in therapeutic angiogenesis. Hind limb ischemia was induced in one leg of each mouse by excision of the femoral

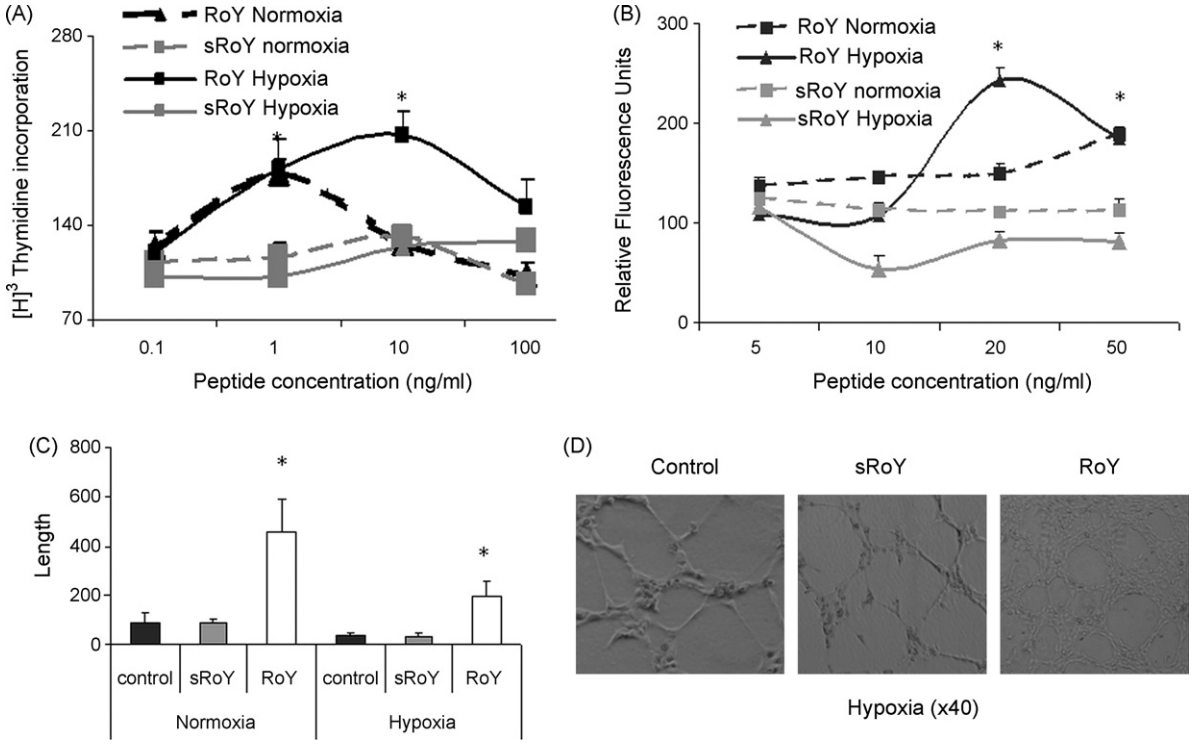

Fig. 2 – (A) Proliferation of endothelial cells obtained after incubation with different concentrations of RoY peptide under hypoxic and normoxic conditions. The percent of thymidine uptake in cells incubated with peptide over control of cells without peptide, was significant ($^{*}p < 0.05$) in both normoxic and hypoxic conditions at 1 ng/ml. Under hypoxic conditions, RoY peptide at 10 ng/ml induced significant ($^{*}p < 0.05$) two-fold increase in thymidine uptake. sRoY did not induce cell proliferation. Values are means ± S.D. of four different experiments using four different cords. (B) Migration of endothelial cells incubated with RoY peptide under hypoxic and normoxic conditions. The net relative increase in migration of endothelial cells was induced under hypoxic and normoxic conditions by 50 ng/ml RoY peptide ($^{*}p < 0.05$) and not by the scrambled form. Twenty nanograms per milliliter of RoY peptide induced significantly greater increase only under hypoxic conditions ($^{*}p < 0.0001$). Values are means ± S.D. of four different experiments. (C) Effect of RoY peptide and sRoY (10 ng/ml for 24 h) on tube formation under normoxic and hypoxic conditions in comparison to untreated cells. RoY peptide significantly increased the length of network of connected cells vs. control ($^{*}p < 0.05$). Values are means ± S.E.M. of three different experiments. (D) Representative images (×40) of endothelial cell tube formation of control untreated endothelial cells, and with the addition of sRoY and RoY peptides under hypoxic conditions.

artery that resulted in ischemia and decreased blood perfusion. We studied in this model the effect of a single local administration of the peptides in restoring blood perfusion using a laser Doppler imager.

The results indicated that a single intramuscular administration of RoY peptide significantly alleviated hind limb ischemia. Fig. 3A summarizes the findings for concentrations of 0.1 and 1 μg RoY peptide and 1 μg scrambled control peptide (sRoY) compared to PBS. Excision of the femoral artery in one leg of each mouse reduced its blood perfusion to half the normal values relative to the non-operated leg on day of operation. Values of relative perfusion obtained by laser Doppler ranged from 0.4 to 0.47. Seven days later, the relative perfusion values of the treated mice with either RoY peptide or sRoY did not increase significant blood perfusion compared to mice injected with PBS and were in the range of 0.6–0.75. Highly significant results ($p < 0.0001$) were obtained on days 14 and 21 with both 1 μg RoY (mean perfusion 85% on day 14 and 92% on day 21) and 0.1 μg RoY (mean perfusion, 83 and 84%, respectively). Scrambled peptide induced a non-significant 72% perfusion compared to spontaneous control PBS (60 and 63%, respectively). The laser Doppler imager scans shown in Fig. 3B were obtained on day 21 in RoY- and PBS-treated mice.

3.4. *Histology*

Histological examination was performed on operated ischemic limb tissues obtained at different times after treatment and compared to controls. Since the muscle samples were taken from the same areas in both the control and treated mice, the difference in vessel counts represents the effect of the peptide. Fig. 4A shows the mean number of vessels in cross-sectional areas of 10 individual fields per sample on postoperative days 7, 14 and 21. RoY peptide treatment significantly increased ($p < 0.05$) the number of capillaries compared to PBS. The representative illustration in Fig. 4B demonstrates the increase in the number of capillaries (density) in the RoY-treated group.

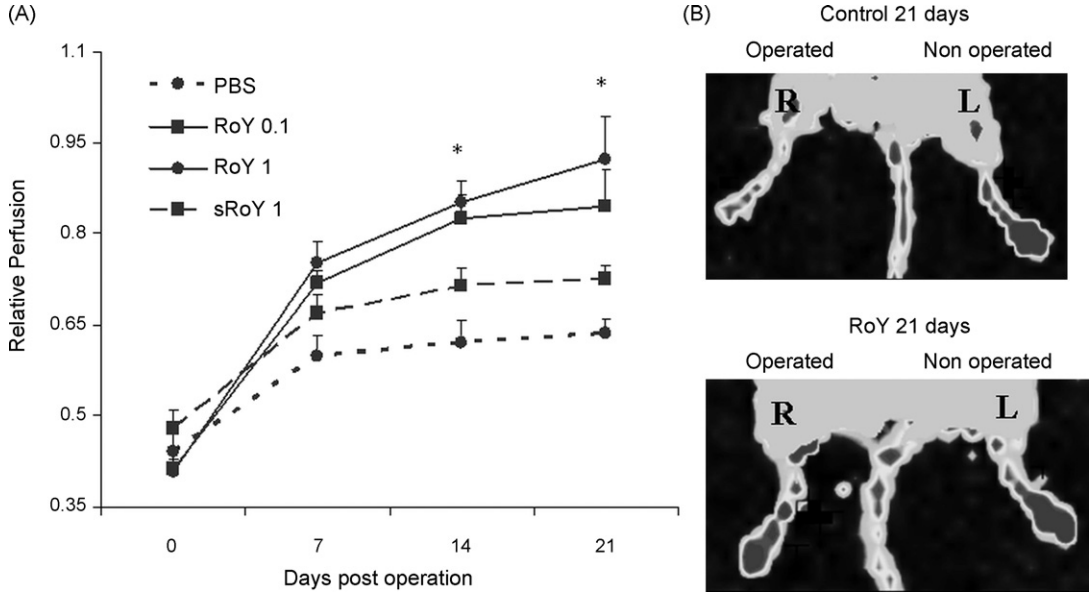

Fig. 3 – Therapeutic angiogenesis of RoY peptide in a mouse hind limb ischemia model. RoY peptide and its scrambled form (sRoY) were injected intramuscularly 1 day after femoral artery excision at a concentration of 0.1 or 1.0 μg/mouse (in a total of 16 mice per group). PBS was used as control. Blood perfusion was measured with a laser Doppler analyzer immediately after surgery and 7, 14 and 21 days later in three different experiments. (A) RoY peptide significantly alleviated hind limb ischemia on days 14 and 21 post-operation ($^{*}p < 0.0001$) compared to PBS. sRoY induced a non-significant increased perfusion compared to control PBS. Values are means ± S.E.M. (B) The images of the laser Doppler show the non-operated (L: left) and operated treated leg (R: right) at 21 days post-operation of RoY treated vs. PBS control mice.

3.5. *Identification of the receptor on endothelial cells that bind RoY peptide*

In order to identify the protein-binding receptor on endothelial cells that binds RoY peptide, we precipitated endothelial cells lysates with RoY peptide and analyzed the precipitated protein.

Fig. 5A shows a polyacrylamid gel electrophoresis of the precipitated cell lysates with RoY peptide under both normoxic and hypoxic conditions. A major band was identified at 78 kDa. To confirm that this band was indeed the peptide-binding protein, we prepared a Western blot membrane and stained it with biotinylated RoY peptide (Fig. 5B). We then cut the major protein band from the polyacrylamide gel and analyzed it by mass spectroscopy. Two repeated analyses of precipitated cell lysates (normoxic and hypoxic) were identified by mass spectroscopy as GRP78 protein. This finding was further confirmed by staining a similar nitrocellulose membrane with anti-GRP78 antibody (Fig. 5C).

In order to confirm that RoY peptide binds to membrane GRP78, we studied by FACS analysis on intact endothelial cells, the inhibition of anti-GRP78 binding in the presence of increasing concentrations of RoY peptide. In three different experiments, increasing concentrations of RoY peptide inhibited binding of anti-GRP78 antibody that was added to endothelial cells under hypoxic conditions. As can be seen in Fig. 5D, representing one of the experiments, a 10 μg of RoY peptide inhibited binding membranous GRP78 from 89.7 to 21.2% while no inhibition resulted by addition of the scrambled peptide.

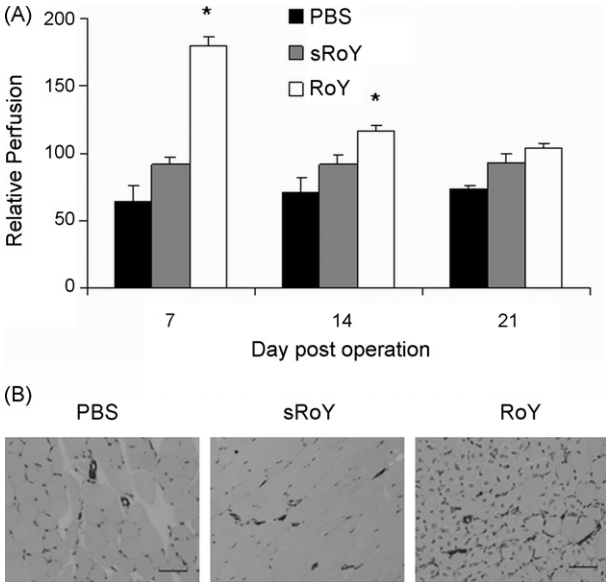

Fig. 4 – Histological assessment of angiogenesis in the ischemic hind limb treated with RoY peptide. (A) Factor VIII staining was used to immunohistochemically quantify mean capillary density in mouse hind legs treated with 1.0 μg RoY peptide compared to control PBS-injected mice on days 7, 14 and 21 post-femoral artery excision. Mean capillary density was significantly higher ($^{*}p < 0.05$) in the limbs of the RoY-treated mice. Error bars represent SEM. (B) Stained sections from limb of PBS, sRoY and RoY treated mice 21 days after surgery. The ruler bar equals 100 μm.

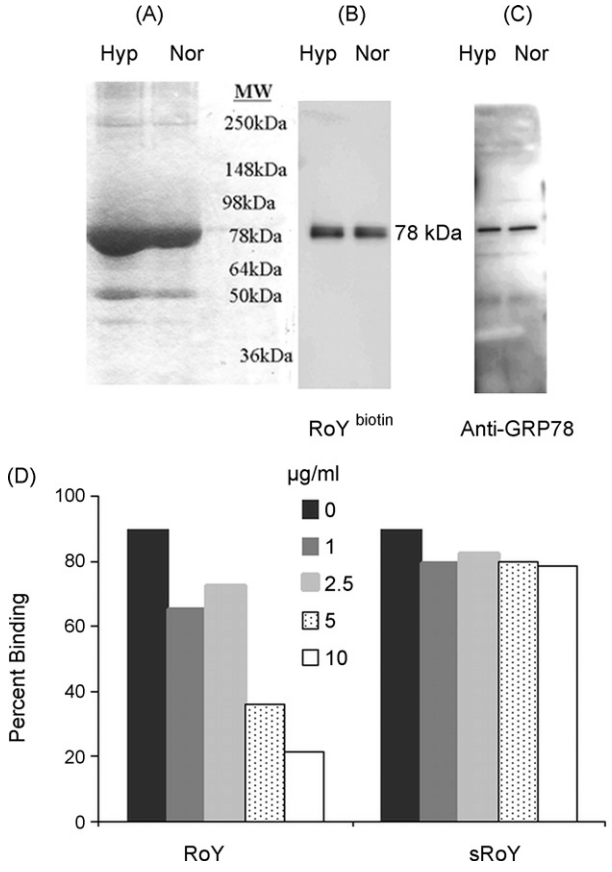

Fig. 5 – Determination of RoY-binding protein on endothelial cells under normoxic or hypoxic conditions. (A) Endothelial cells lysates precipitated with RoY peptide were analyzed by gel electrophoresis. Gel stained with Coomassie blue showed a major protein band at 78 kDa that was cut and identified as GRP78 protein by mass spectroscopy. (B) Confirmation of peptide-binding protein by Western blot membrane staining with biotinylated RoY peptide. (C) Confirmation of mass spectroscopy identification of GRP78 protein by staining a nitrocellulose membrane with anti-GRP78 antibody. (D) FACS analysis demonstrating inhibition of anti-GRP78 binding to intact endothelial cell membranes by increasing concentrations of RoY peptide under hypoxic conditions while no inhibition resulted by addition of sRoY.

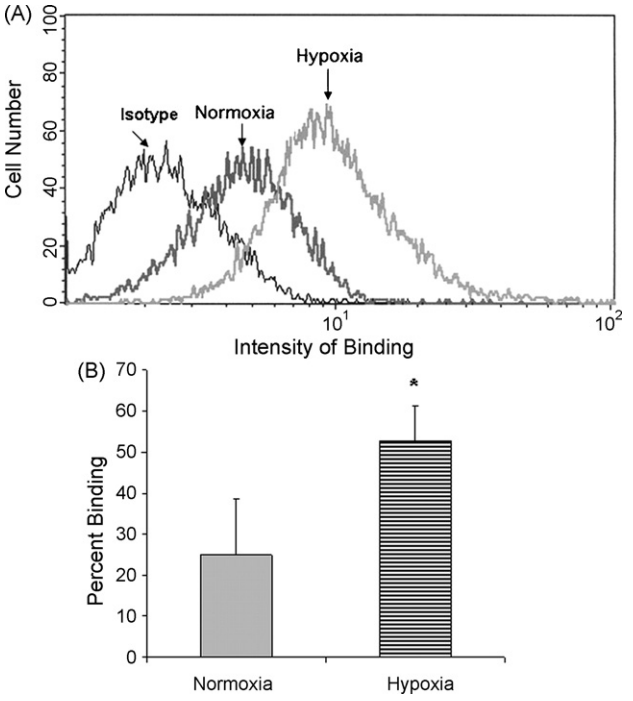

Fig. 6 – GRP78 receptor on endothelial cells. (A) FACS analysis of anti-GRP78 binding to endothelial cells under normoxic and hypoxic conditions. Binding of anti-GRP78 was greater in cells exposed to 5 h hypoxia. IgG1-FITC was used as isotype control. (B) Increased binding of anti-GRP78 to endothelial cells under hypoxic conditions compared to normoxic conditions. Values represent means ± S.D. of percent binding of endothelial cells from 10 different cords ($^{*}p < 0.05$).

Subsequent FACS analysis of the endothelial cells with anti-GRP78 corroborated the increase both the percent and intensity of the receptor on endothelial cell membranes in complete medium under hypoxic conditions (Fig. 6A). As seen in Fig. 6B, the anti-GRP78 binding to endothelial cells originated from 10 different cords, increasing from a mean percentage of 30 ± 13% under normoxic conditions to 52.8 ± 8.4% after 5 h of hypoxia.

4. Discussion

The present study demonstrates that RoY, a synthetic 12 amino-acid peptide, manifests angiogenic activity under hypoxic conditions. Proliferation of endothelial cells incubated with RoY peptide under hypoxia increased significantly by two-fold, probably as a result of a higher binding to the hypoxic cells as was also demonstrated by FACS analysis. Accordingly, RoY was initially identified from a phage display peptide library screening on endothelial cells under hypoxic conditions, previously designated as peptide YR [10]. RoY peptide also as chemoattractant in a migration chamber, induced significant increase in endothelial cell migration under hypoxic conditions at 20 ng/ml. Under normoxic conditions a higher concentration of 50 ng/ml was needed to obtain significant migration. Tube formation, the ability to form network of connected cells in tube-like structures is a typical response to angiogenic stimulation. RoY peptide induced tube formation equally under both normoxic and hypoxic conditions.

In order to assess the therapeutic activity of RoY peptide in ischemic tissue, we used the mouse hind limb ischemia model that was used previously to assess therapeutic activity of angiogenic factors [12–16]. We found that a single local administration of RoY peptide to the ischemic limb restored blood perfusion to normal values. The specificity of the therapeutic activity was corroborated by the lack of response to the scrambled peptide (sRoY). Morphometric analysis of capillary density, performed at the same time points selected

for *in vivo* laser Doppler blood flow analysis, confirmed that the histological sequence of neovascularization corresponded to the recovery in perfusion.

Given that RoY peptide does not induce VEGF receptor expression [10], its binding to the GRP78 receptor on endothelial cell membranes suggests a different mechanism of angiogenesis. GRP78, also known as immunoglobulin heavy chain-binding protein (BiP), is a 78 kDa glucose-regulated heat shock protein involved in numerous intra- and intercellular processes, including protein synthesis and folding. It is normally expressed in the lumen of the endoplasmic reticulum of almost all cell types [17,18]. However, its expression and presence on surface membranes increases under conditions of stress [19–21].

In our study, GRP78 is over expressed in hypoxia *in vitro* or ischemic tissue *in vivo*, therefore it is possible that increased RoY peptide binding to cells under hypoxia leads to increased angiogenic activity.

Recently, it was found that binding to a segment of membrane GRP78 by antibodies from prostate patient serum, induced prostate cancer cell proliferation [22]. In our study RoY peptide binding to membrane GRP78 on endothelial cells also induced endothelial cell proliferation. In contrast, peptides derived from Kringle 5 (K5) of human plasminogen, inhibited angiogenesis by inducing apoptosis of proliferating endothelial cells [23]. It is therefore possible that different peptides bind different epitopes on membrane GRP78 resulting in different signaling. Although HIF-1 is an important motif in response to hypoxia following VEGF induction, it does not appear to play a role in the induction of GRP78 under chronic hypoxia. No recognizable HIF-1-binding motif is present in the sequence of the GRP78 promoter [24–26].

The present study expands our understanding on VEGF-independent angiogenesis in stress states. We describe a novel peptide that induces angiogenesis under hypoxic conditions and alleviates hind limb ischemia by activating the stress protein GRP78 receptor on endothelial cells. In addition, these data provide experimental evidence that GRP78 is an angiogenic receptor on hypoxic endothelial cells.

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