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Systemic Administration of Thrombin Peptide TP508 Enhances VEGF-Stimulated Angiogenesis and Attenuates Effects of Chronic Hypoxia

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Key Words
Therapeutic angiogenesis · Vascular endothelial growth factor · Thrombin peptide · Rusalatide acetate · Endothelial dysfunction · Hypoxia · Endothelial cells

Abstract
Revascularization of chronic wounds and ischemic tissue is attenuated by endothelial dysfunction and the inability of angiogenic factors to stimulate angiogenesis. We recently showed that TP508, a nonproteolytic thrombin peptide, increases perfusion and NO-dependent vasodilation in hearts with chronic ischemia and stimulates NO production by endothelial cells. In this study, we investigated systemic in vivo effects of TP508 on VEGF-stimulated angiogenesis in vitro using aortic explants in normoxic and hypoxic conditions. Mice were injected with saline or TP508 and 24 h later aortas were removed and cultured to quantify endothelial sprouting. TP508 injection increased endothelial sprouting and potentiated the in vitro response to VEGF. Exposure of control explants to hypoxia inhibited basal and VEGF-stimulated endothelial cell sprouting. This effect of hypoxia was significantly prevented by TP508 injection. Thus, TP508 systemic administration increases responsiveness of aortic endothelial cells to VEGF and diminishes the effect of chronic hypoxia on endothelial cell sprouting. Studies using human endothelial cells in culture suggest that protective effects of TP508 during hypoxia may involve stimulation of endothelial cell NO production. These data suggest potential clinical benefit of using a combination of systemic TP508 and local VEGF as a therapy for revascularization of ischemic tissue.

Introduction

TP508 is a 23 amino acid synthetic peptide representing residues 508–530 of human prothrombin which was identified as a potential receptor-binding domain based on competition for high-affinity thrombin binding to fibroblasts [1]. In contrast to thrombin, TP508 has no enzymatic activity and does not promote or interfere with blood coagulation [1]. Studies have shown that TP508 accelerates healing of dermal wounds [2–4], fractures [5] and bone defects [6, 7]. TP508 is being evaluated in human clinical trials as a therapeutic drug for treatment of diabetic foot ulcers [8] and for acceleration of fracture

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repair [9]. TP508 acceleration of healing is associated with stimulation of early revascularization in dermal and bone injury models [2, 3, 5, 10]. From these studies, however, it is not clear whether TP508 stimulated angiogenesis by itself or promoted angiogenic responses to other factors present in the injured tissue.

VEGF is an important angiogenesis factor that is produced by hypoxic cells during tissue injury to stimulate and direct angiogenesis [11, 12]. A number of studies have shown that VEGF-stimulated endothelial angiogenic responses in vitro [13–17] and angiogenesis in vivo [18, 19] require activation of endothelial nitric oxide synthase (eNOS). Although VEGF administration increases revascularization of ischemic tissue in experimental animal models, only limited benefit was achieved with VEGF therapeutic angiogenesis in clinical trials in patients with myocardial ischemia [20–22]. Thus, this limited effect of VEGF in patients with coronary artery disease suggests decreased endothelial cell responsiveness to VEGF in chronically hypoxic tissues. We have shown that exposure of human endothelial cells to chronic hypoxia attenuates VEGF-stimulated signaling and angiogenic responses [23]. We recently demonstrated that TP508 activates eNOS and stimulates production of NO in human endothelial cells [23]. Using a pig model of chronic myocardial ischemia, we demonstrated that TP508 increased myocardial perfusion and function, and restored NO-dependent vasodilatation and NO production in arterioles isolated from ischemic myocardial regions [24]. More recently, it has been shown that TP508 decreased infarct size, improved NO-dependent microvascular function and increased activation of eNOS following acute myocardial ischemia-reperfusion injury in normal [25], hypercholesterolemic [26] and diabetic pigs [27]. These studies led us to hypothesize that TP508 protects endothelial cells from hypoxia-induced loss of endothelial function, and potentiates responsiveness of vascular endothelial cells to VEGF.

The purpose of this study was to examine systemic effects of TP508 on VEGF-stimulated angiogenesis in vitro using aortic explants and to determine whether TP508 administrated in vivo protects endothelial cells against effects of chronic hypoxia. Our results demonstrate that TP508 intravenous injection increases endothelial cell sprouting and enhances VEGF-stimulated sprouting from aortic explants isolated from mice 24 h after injection. Moreover, chronic hypoxia markedly inhibits basal and VEGF-induced endothelial cell sprouting, but TP508 injection protects endothelial cells from this hypoxic effect. These results suggest potential therapeutic benefit of a combination of VEGF and TP508 for myocardial revascularization or treatment of peripheral artery disease.

Materials and Methods

Reagents

Thrombin peptide TP508 (AGYKPDEGKRGDACEGDSGGPFV, also known as rusalatide acetate CAS No. 87455-82-6) was synthesized and purified by American Peptide Company (Sunnyvale, Calif., USA). Human recombinant VEGFA165 was purchased from R&D systems (Minneapolis, Minn., USA). Matrigel Matrix (phenol red free) was obtained from (BD Biosciences, Bedford, Mass., USA). L-arginine, $NaNO_2$, and NaI were purchased from Sigma (St Louis, Mo., USA).

Animals

All experiments were approved by the UTMB Institutional Animal Care and Use Committee. Twelve- to 15-week-old ICR male mice (Charles River Laboratories, Wilmington Mass., USA) were housed (5 per cage) in a controlled environment with a 12-hour light/dark cycle, with temperature maintained at 21 ± 0.5°C and a room humidity of 50 ± 20%. Water and chow were provided ad libitum. Mice were placed in a restrainer and their tail veins were injected with a single 500-μg dose of TP508 in 100 μl of saline or with 100 μl of saline alone (placebo control). A single injection of TP508 (500 μg/mouse) was chosen for these studies based on dose response studies of TP508 injection on ex vivo aortic endothelial cell sprouting and in vivo biological effects where injection of 500 μg/mouse showed maximal effects [manuscript in preparation]. After 24 h mice were euthanized by CO_2 inhalation and aortas were removed as previously described [28, 29].

Mouse Aortic Explant Angiogenesis Assay

Angiogenic endothelial cell sprouting was studied by culturing mouse aortic explants on Matrigel Matrix (BD Biosciences) as described previously [28, 29]. Twenty-four hours postinjection, thoracic aortas were isolated from TP508- or saline-administrated mice and transferred to culture dishes containing cold endothelial basal medium (EBM; Lonza, Walkersville, Md., USA). The periaortic fibroadipose tissue was removed under a dissecting microscope and aortas were rinsed with cold EBM and cut transversely to create 1 mm aortic rings (~10 per aorta). Aortic rings were cut, opened and the inner endothelial surface was placed directly on Matrigel Matrix coated wells of 24-well plates. Aortic explants were cultured in endothelial growth medium which contains EBM supplemented with 5% fetal bovine serum and SingleQuots (Lonza) in 5% CO_2 at 37°C. After 24 h of culture, aortic explants from control or TP508-injected mice were stimulated with VEGF (50 ng/ml) or vehicle and cultured in normoxia. To determine the effects of hypoxia, aortic explants from control and TP508-injected mice were incubated with or without VEGF under hypoxic conditions (1% O_2, 5% CO_2) for 2 days and then switched back to normoxia. Endothelial cell sprouting was monitored daily by inverted phase-contrast microscopy (Nikon, Tokyo, Japan) and images were captured using a Spot RT camera

with Spot Advance image capture software (Diagnostic Instruments Inc., Sterling Heights, Mich., USA) at ×40 and ×100 magnification.

Quantification of Endothelial Sprouting

Image analysis and quantification for the area occupied by sprouting endothelial cells and maximal endothelial cell migration from aortic explants edges was performed using MetaMorph software (Molecular Devices, Downingtown, Pa., USA). For each experiment, we isolated aortas from three controls and from three TP508-injected mice. Aortic sprouting was quantified from triplicate culture wells containing a total of 6 aortic explants per experimental condition. The area of endothelial sprouting was normalized to the perimeter of aortic explants visualized in the field of observation. The area of sprouting from control mouse explants cultured in normoxia for 5 days was expressed as a value of 1.0. For maximum endothelial cell migration we measured the longest distance of migrated cells from the edges of aortic explants in 3 different regions from each explant.

Endothelial Cell Culture

Human coronary artery endothelial (HCAE) cells from Lonza were cultured in endothelial growth medium (EBM supplemented with 5% FBS and SingleQuots; Lonza) in 5% CO_2 at 37°C. Two-day postconfluent HCAE cells, between passages 4 and 6, were cultured in normoxic or hypoxic (1% O_2, 5% CO_2 at 37°C) conditions and assayed for effects of hypoxia on VEGF expression, viability and NO production.

Real-Time PCR

Quantitative SYBR Green real-time PCR was used to determine the expression of human VEGF mRNA. HCAE cells were cultured on 6-well plates. Two-day postconfluent cells were treated with TP508 (50 μg/ml) or vehicle and cultured in normoxic or hypoxic conditions for 24 h. Total RNA was isolated using RNAqueous kits (Ambion, Austin, Tex., USA). Isolated mRNA samples were coded for blinded analysis. Total RNA was reverse transcribed using Taqman Reverse Transcription Reagents Kits (ABI). Quantitative PCR amplifications were done using 2 μl of cDNA in a total volume of 25 μl using SYBR Green PCR Master Mix (ABI) on an ABI Prism 7000 Sequence Detection System. Primers for human VEGFA were sense: TAC CTC CAC CAT GCC AAG TG and antisense: GCG CTG ATA GAC ATC CAT GAA C. These primers recognize common sequence for all splice variant transcripts of human VEGFA. Reverse-transcribed cDNA cycle threshold values were calculated from triplicate sample determinations and normalized to 18S RNA.

NO Assay

Confluent HCAE cells were cultured on 24-well plates under normoxic or hypoxic conditions for 24 h. Normoxic and hypoxic cells were then stimulated with TP508 (50 μg/ml) or VEGF (50 ng/ml) or vehicle (control) in the same conditions. After 24 h of stimulation, the medium was replaced with EBM containing 200 μM L-arginine (250 μl/well) and all cells were assayed for NO production during 1 h normoxic incubation. Supernatants were collected and analyzed for NO concentrations using a chemiluminescence NO analyzer (Model 270B, Sievers Instruments, Boulder, Colo., USA) as previously described [30]. The analyzer was calibrated for each experiment using a sodium nitrite standard curve. NO concentration in the EBM containing L-arginine was used as a background value and was subtracted from sample values to determine the amount of NO released from cells.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine if there was a significant difference among groups. A pair-wise comparison of groups was then done using the Tukey test. $p < 0.05$ was regarded as significant.

Results

TP508 Systemic Injection Increases Endothelial Sprouting from Aortic Explants

Stimulation of angiogenesis leading to tissue revascularization is required for normal wound healing after injury or surgical procedures [31]. The aortic sprouting assay is a well-established model for studying the effects of different factors on angiogenesis in vitro [28, 29]. We used this model to determine the systemic effects of TP508 administered in vivo on angiogenic endothelial cell functions. Aortic explants were isolated from mice 24 h after TP508 or saline injection and cultured on Matrigel Matrix. As shown in figure 1a, endothelial cell sprouting was observed in aortic explants from control and TP508-injected mice after 4 days in culture. Explants from TP508-injected mice showed greater sprouting than controls at day 4 (fig. 1a). These sprouting endothelial cells grew progressively to form a branching network by day 5 characteristic of endothelial cells cultured on Matrigel (fig. 1a). Morphometric analysis of the area occupied by endothelial cells and maximal endothelial cell migration was significantly greater in aortic explants isolated from TP508-injected mice than in control explants on days 4, 5 and 6 (fig. 1b). For example, TP508-injected mouse aortic explants had a 6.4-fold increased area and 3.1-fold increased maximal migration of sprouting compared to explants from control mice at day 5 (fig. 3a). Thus, a single TP508 intravenous injection increased endothelial sprouting from aortic explants measured several days later.

TP508 Systemic Injection Potentiates VEGF-Stimulated Angiogenesis

VEGF is a key angiogenesis factor produced during tissue injury to stimulate and direct angiogenesis [11, 12]. Thus, we examined the effect of in vivo systemic TP508 administration on angiogenesis stimulated by VEGF in vitro using the aortic explants. As expected, VEGF treatment of aortic explants from control mice resulted in in-

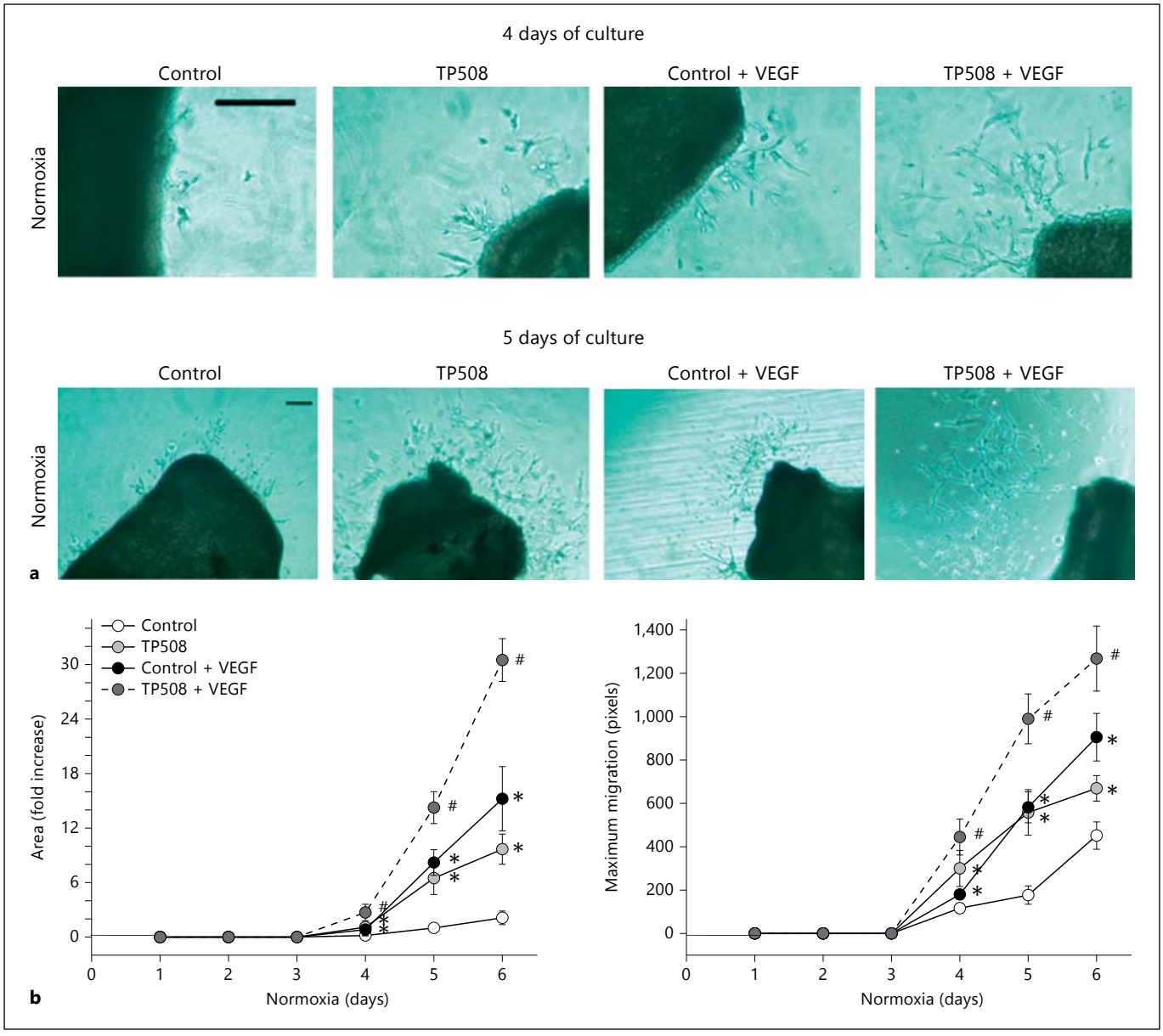

Fig. 1. TP508 injection increases endothelial sprouting and potentiates VEGF-stimulated angiogenesis. Aortas were isolated from mice 24 h postintravenous injection of saline (control) or TP508 (500 μg/100 μl). Aortic explants were cultured on Matrigel Matrix for 6 days under normoxic conditions in growth medium without or with VEGF (50 ng/ml) and photographed daily. **a** Representative images of endothelial sprouting from explants after 4 and 5 days of culture. Four- and 5-day images were taken under ×100 and ×40 magnifications, respectively. Scale bar = 200 μm. **b** Digital image quantification of area and maximal migration of cell sprouting from aortic explants (days 1–6). Data are expressed as a mean ± SD; n = 4, * $p < 0.05$ compared to control, # $p < 0.05$ compared to control + VEGF.

creased area and migration of sprouting at all examined days compared to control, untreated explants (fig. 1a, b). We observed an 8.2- and 3.3-fold increased area and migration of sprouting, respectively, after 5 days culture in aortic explants from control mice in response to VEGF (fig. 3a, normoxia). This VEGF-stimulated sprouting from control explants at day 5 was comparable to the sprouting from aortic explants isolated from TP508-injected mice without VEGF stimulation (fig. 3a, normoxia). Importantly, VEGF stimulation of aortic explants isolated from TP508-injected mice showed significantly greater endothelial sprouting compared to aortic sprouting in re-

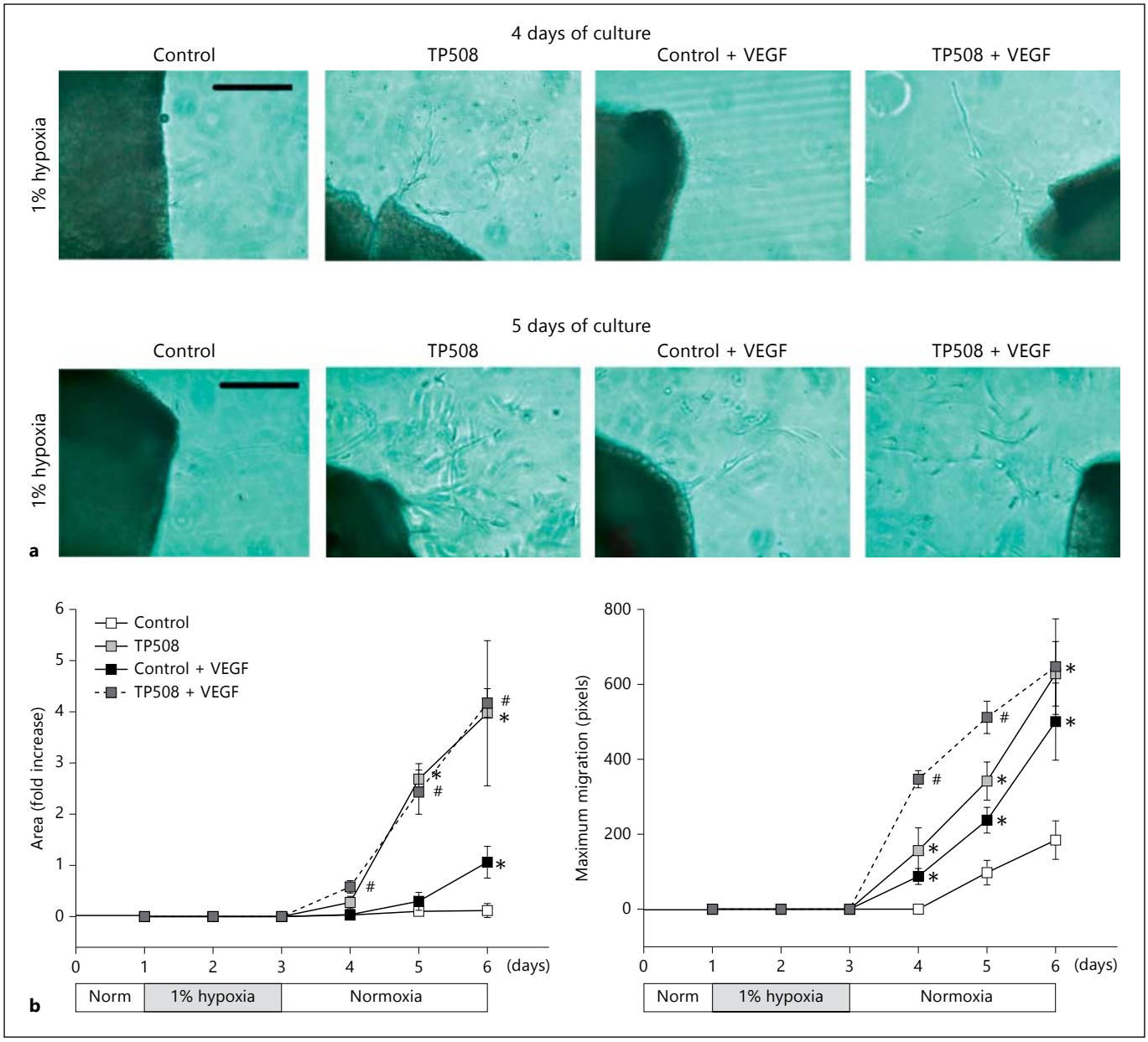

Fig. 2. Effect of chronic hypoxia on endothelial cell sprouting. Aortas were isolated from saline (control) or TP508-injected (500 μg/100 μl) mice and aortic explants were cultured on Matrigel Matrix. Aortic explants from control or TP508-injected mice were stimulated with or without VEGF (50 ng/ml; control + VEGF and TP508 + VEGF, respectively) and exposed to hypoxia (1% O_2) for 2 days and returned to normoxia. **a** Representative photographs of cell sprouting from aortic explants after 4 and 5 days of culture are shown (magnification ×100). Scale bar = 200 μm. **b** Digital image quantification of area and maximal migration of cell sprouting from aortic explants. Data are expressed as a mean ± SD; n = 4, * $p < 0.05$ compared to control, # $p < 0.05$ compared to control + VEGF.

sponse to VEGF from control mice on days 4, 5 and 6 (fig. 1a, b). These results demonstrate that in addition to promoting endothelial sprouting, TP508 injection also increases responsiveness of endothelial cells from aortic explants to VEGF-stimulated angiogenesis in vitro.

TP508 Injection Diminished Effects of Hypoxia on Angiogenesis

We have shown that exposure of human coronary endothelial cells to chronic hypoxia (1% O_2) attenuates endothelial cell angiogenic responses [23]. The aortic angio-

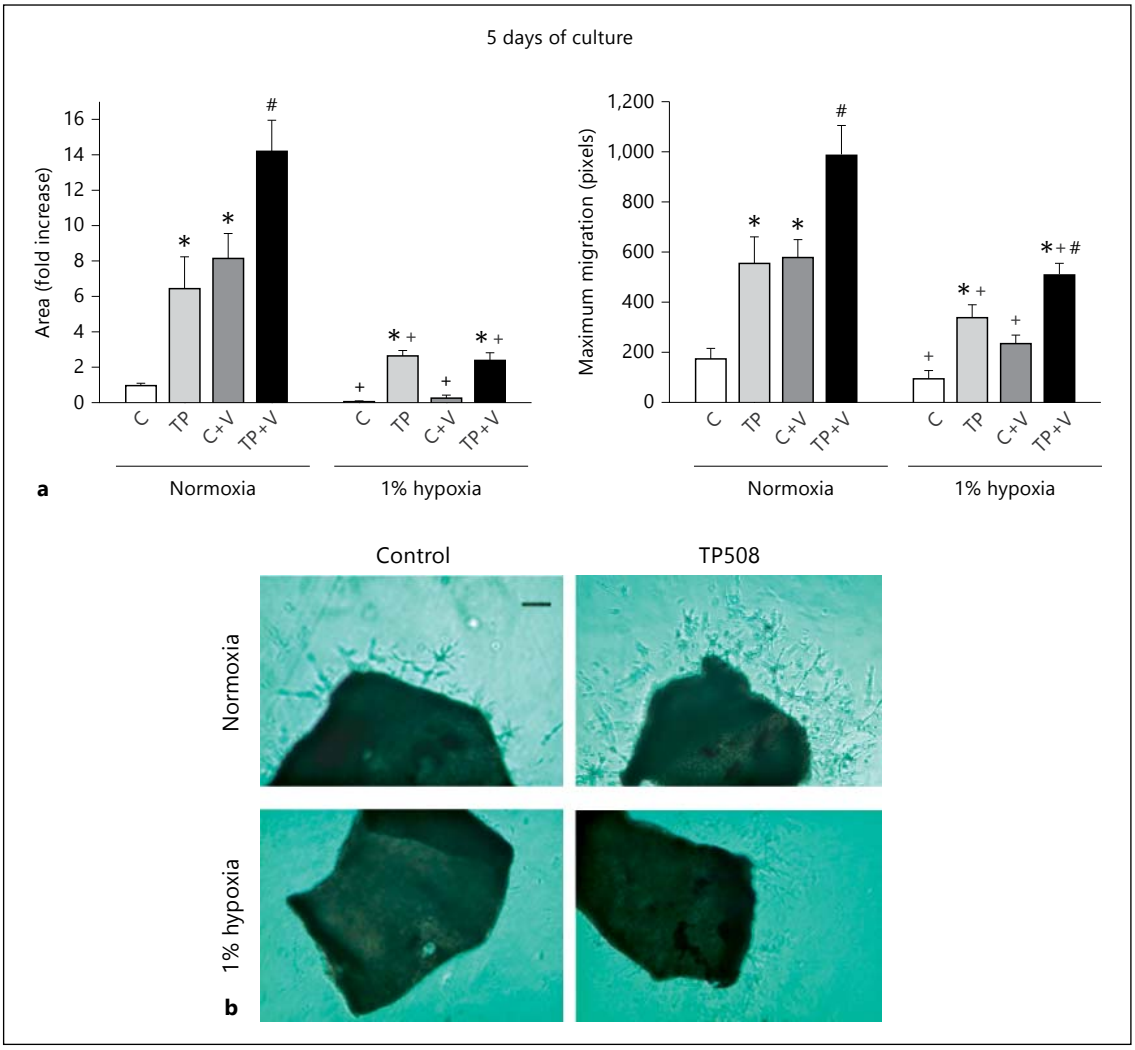

Fig. 3. Comparison of endothelial cell sprouting after 5 days of culture in normoxic and hypoxic conditions. **a** Area and migration of endothelial sprouting was quantified after 5 days and statistically analyzed for the effect of hypoxic exposure. Data are expressed as mean ± SD; n = 4; * $p < 0.05$ compared to control in normoxia, # $p < 0.05$ compared to control + VEGF, + $p < 0.05$ compared to normoxia for each treatment. C = Control; TP = TP508; V = VEGF. **b** Effect of TP508-injection on hypoxia-induced attenuation of sprouting. Representative photographs of cell sprouting from aortic explants isolated from control and TP508-treated mice cultured in normoxic or hypoxic conditions for 5 days are shown (magnification ×40). Scale bar = 200 μm.

genic sprouting assay allows us to evaluate ex vivo effects of hypoxia on angiogenic responses of endothelial cells from intact vessel walls. This model also allows us to determine whether TP508 delivered in vivo can protect endothelial cells against effects of hypoxia.

Aortic explants isolated from control and TP508-injected mice were cultured for 24 h in normoxia to allow the explants to attach to the Matrigel and then switched to hypoxia (1% O_2). In preliminary experiments we observed that continued culture of aortic explants under hypoxia completely inhibited endothelial sprouting, but if explants were cultured under hypoxia (1% O_2) for 2 days and then returned to normoxia, measurable sprouting was observed. Explant exposure to hypoxia for 2 days markedly decreased the area of sprouting compared to the same explants cultured continuously in normoxia (fig. 2a, b, 3a, b). Moreover, VEGF-stimulated sprouting was suppressed during hypoxic exposure (fig. 2, 3a). At day 5 of culture, the area and maximal migration of endothelial sprouting in VEGF-stimulated control explants exposed to hypoxia was reduced 27- and 2.5-fold, respectively, compared to VEGF-stimulated sprouting in nor-

moxia (fig. 3a). Hypoxia also significantly decreased endothelial sprouting in aortic explants isolated from TP508-injected mice compared to normoxic culture (2.3- and 1.6-fold; fig. 3a, b), however, the area and cell migration of sprouting at day 5 of culture was similar to sprouting from explants isolated from control mice cultured in normoxia (fig. 3a). In contrast to normoxia, there was no significant difference in area of sprouting from aortic explants isolated from TP508-injected mice in response to VEGF stimulation when they were exposed to hypoxia (fig. 3a). However, we observed a significant increase in maximal migration of endothelial cells in response to VEGF stimulation from hypoxic explants isolated from TP508-treated mice compared to explants from control mice (at day 4 and 5 culture; fig. 3a). These results show that exposure of aortic explants to chronic hypoxia markedly attenuates basal and VEGF-stimulated angiogenesis. Moreover, explants from TP508-injected mice that were exposed to hypoxia had levels of sprouting similar to that seen in control mouse explants cultured in normoxia. Therefore, TP508 injection protected endothelial cells from hypoxic effects.

Effect of TP508 Treatment on Expression of VEGF in HCAE Cells

To gain a better understanding of how TP508 may exert its effects on endothelial cells in vivo, we examined effects of TP508 on HCAE cells in vitro. We first determined TP508 effects on VEGF expression since increased VEGF production might explain the in vivo effects of TP508. Real-time PCR experiments showed that under both normoxic and hypoxic (1% O_2) conditions, TP508 did not cause significant changes in VEGF mRNA expression levels during 24 h culture. Exposure of cells to hypoxia for 24 h increased VEGF mRNA expression 6.8 ± 1.3- and 7.5 ± 1.8-fold in control and TP508-treated cells, respectively (fig. 4b). These results showed that TP508 stimulation did not significantly affect the VEGF mRNA levels in normoxic or hypoxic cells. The concentrations of VEGF protein secreted into supernatants were also not significantly different between control and TP508-treated cells (data not shown). These results suggest that the in vivo effects of TP508 on endothelial sprouting are not likely to be due to TP508 changes in endothelial cell VEGF expression.

Effects of Chronic Hypoxia on HCAE Cell Viability and NO Production

To determine whether hypoxia-induced inhibition of endothelial sprouting from aortic explants was due to endothelial cell death, we exposed HCAE cells to hypoxia (1% O_2) for 48 h. Hypoxic cells remained confluent and cell viability was similar to cells cultured in normoxia (fig 4a).

Production of NO by endothelial cells plays an important role in VEGF-stimulated angiogenesis in vivo [18] and in vitro [32]. Therefore, we measured the amount of NO produced by HCAE cells during 1 h incubation, 24 h after stimulation with TP508 or VEGF. As shown in figure 4c, TP508 and VEGF stimulated 115 ± 33 and 111 ± 16 nM of NO production, respectively, compared to 41 ± 5 nM of NO released from control cells (fig. 4c). Exposure of cells to hypoxia for 48 h resulted in attenuation of NO production from control (25 ± 5 nM) and VEGF-stimulated cells (40 ± 17 nM). However, TP508-treatment of hypoxic cells increased NO production approximately 2-fold relative to control normoxic cells (79 ± 13 vs. 41 ± 5 nM; fig. 4c). Thus, hypoxia significantly inhibited VEGF-stimulated NO production, but had no significant effect on NO production induced by TP508. TP508-induced increase in NO production in hypoxic endothelial cells correlates with TP508-stimulated endothelial sprouting from aortic explants exposed to chronic hypoxia. This suggests that in vivo TP508 may have a similar effect on NO production which serves to protect endothelial cells from hypoxia.

Discussion

The present studies were undertaken to determine the in vivo effect of thrombin peptide, TP508, on endothelial cell angiogenic responses under normoxic and hypoxic conditions. To address these questions we injected TP508 intravenously into mice and evaluated the effects of the peptide on angiogenesis using an aortic explant endothelial sprouting assay that mimics several stages of angiogenesis, including endothelial cell sprouting, proliferation, migration and tube-like structure formation [29]. Our results demonstrate that TP508 injection into mice 24 h prior to removal of aortas promotes endothelial sprouting and potentiates VEGF-stimulated endothelial sprouting. Exposure of aortic explants to chronic hypoxia markedly inhibited endothelial sprouting even after VEGF stimulation. However, aortic explants from TP508-injected mice exposed to hypoxia showed increased sprouting similar that observed in aortic explants isolated from control mice cultured in normoxia.

Effective wound repair is dependent on the number of cellular processes including revascularization of injured

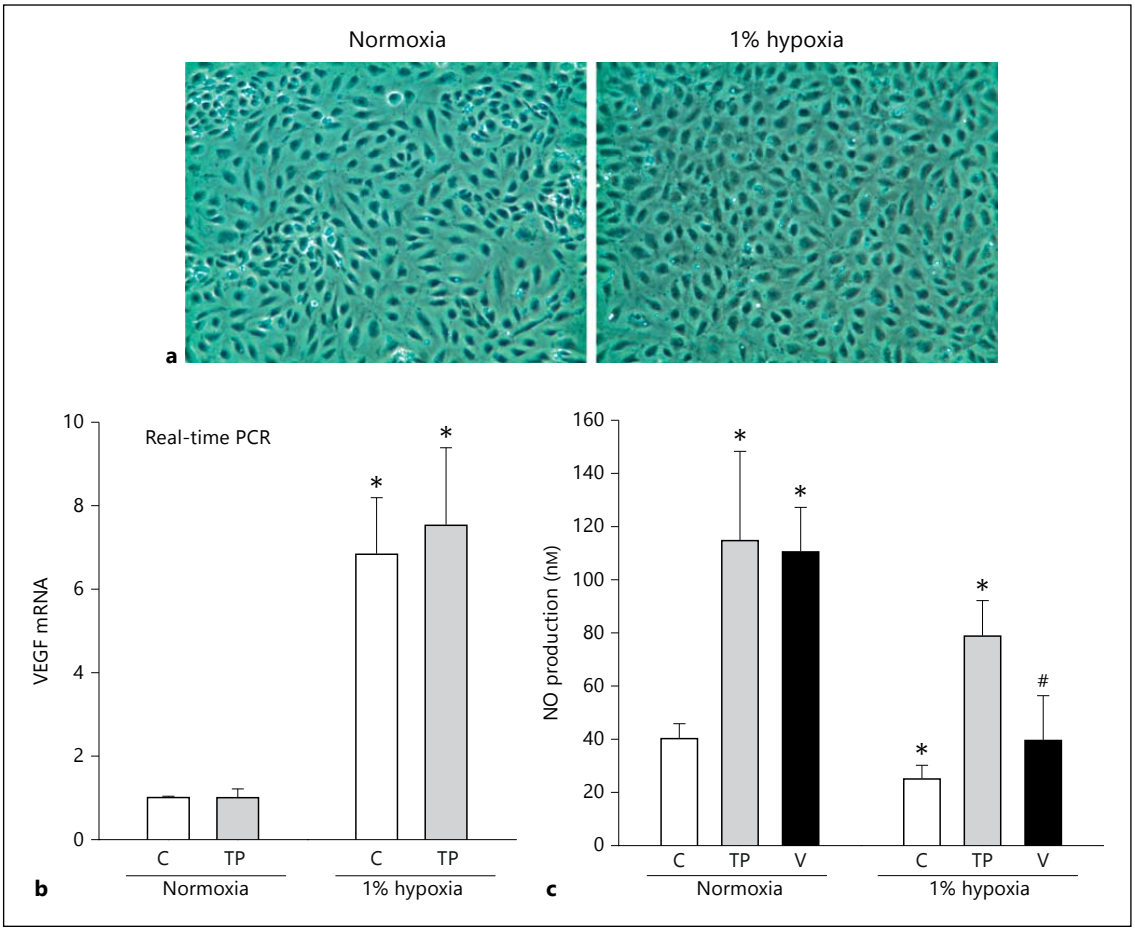

Fig. 4. Effects of TP508 and hypoxia on endothelial cell viability, expression of VEGF and NO production in vitro. **a** Confluent cultures of HCAE cells were maintained in normoxic or hypoxic (1% O_2) conditions for 48 h. Representative phase-contrast images show viability of hypoxic cells comparable to normoxic cells (magnification ×100). **b** Real-time PCR analysis of human VEGF mRNA expression from control and TP508-treated (50 μg/ml) HCAE cells cultured in normoxic or hypoxic conditions for 24 h. Data are expressed as a mean ± SD; n = 4; * $p < 0.01$ compared to control in normoxia. **c** Confluent cultures of HCAE cells were maintained in normoxic or hypoxic (1% O_2) conditions for 24 h. Next, cells were stimulated with TP508 (50 μg/ml) and VEGF (50 ng/ml) or vehicle control in the same conditions. After 24 h, medium was replaced and all cells were cultured in normoxia. Supernatants were collected after 1 h and NO concentration was measured by NO analyzer. Data are expressed as a mean ± SD; n = 4, * $p < 0.01$ compared to control in normoxia, # $p < 0.01$ compared to VEGF in normoxia. C = Control; TP = TP508; V = VEGF.

tissues [31]. TP508 has been previously shown to accelerate tissue revascularization and repair following tissue injury [2, 3, 5, 7, 10]. In a pig model of chronic myocardial ischemia, TP508 injection into the ischemic myocardium increased perfusion and restored myocardial function [24]. This treatment also increased NO-dependent vasodilation, expression of eNOS and NO production in arterioles isolated from ischemic heart regions [24]. We have shown that at the molecular level, TP508 stimulates rapid NO production in primary human endothelial cells [30] and prevents downregulation of eNOS expression in cells exposed to chronic hypoxia [24]. Intravenous injection of TP508 has recently been shown to reduce the infarct area following acute myocardial ischemia-reperfusion injury in normal [25], hypercholesterolemic [26] and diabetic pigs [27]. This systemic effect of TP508 caused us to question whether systemic administration of TP508 could enhance angiogenesis or protect endothelial cells from ischemia.

The aortic sprouting assay is a well-established model for studying the effects of different factors on angiogenesis in vitro [28, 29]. We used this model in a novel manner to determine in vivo effects of systemically injected TP508 on vascular endothelial cell responses to ex vivo

angiogenic factors and hypoxia. To determine whether a single dose of TP508 injected into mice promotes angiogenesis, we isolated aortas from placebo control or TP508-injected mice 24 h after injection and compared endothelial sprouting from aortic explants cultured on Matrigel Matrix. Our data show that TP508 injection significantly increased the area of endothelial sprouting and maximal migration of endothelial cells compared to aortic sprouting from control mouse explants.

VEGF is an endothelial-specific growth factor that is secreted by hypoxic cells at the site of injury to stimulate angiogenesis [11, 12]. We therefore tested whether TP508 injection increases the responsiveness of endothelial cells to VEGF-stimulated angiogenesis. We found that VEGF stimulation of aortic explants isolated from TP508-injected mice showed significantly greater sprouting compared to VEGF-stimulated sprouting from control explants. These results demonstrate that TP508 injection not only promotes endothelial sprouting from aortic explants, but also potentiates responsiveness of aortic endothelial cells to VEGF.

Revascularization of chronic wounds and ischemic tissue is attenuated by endothelial dysfunction. Endothelial dysfunction induced by chronic hypoxia impairs angiogenesis in response to angiogenic factors [23]. In experimental animals, revascularization and recovery of blood flow to ischemic tissue is increased by administration of VEGF, but human clinical trials of therapeutic angiogenesis with VEGF were less successful than anticipated [20, 21, 33]. The differences between results in animals and clinical outcomes are postulated to involve different responsiveness of endothelial cells in young healthy animals used in laboratory studies compared to the endothelial cell responses in patients with chronic myocardial ischemia. Indeed, patients with coronary artery disease have endothelial dysfunction characterized by decreased expression of eNOS in endothelial cells [34, 35] which contributes to attenuation of NO production and defective NO-dependent vasodilation in atherosclerotic vessels [36]. In the present study, we also evaluated the effect of chronic hypoxia on angiogenesis using aortic explants. Our preliminary results showed that continued culture of aortic explants under hypoxia (1% O_2) completely inhibited endothelial sprouting, thus we exposed the aortic explants to hypoxia for 2 days followed by culture in normoxia. Exposure of aortic explants to hypoxia for 2 days inhibited endothelial cell sprouting and also markedly diminished VEGF-stimulated aortic sprouting compared to normoxia. These results are consistent with our previous report showing that chronic hypoxia decreases VEGF-stimulated signaling and angiogenic responses of endothelial cells [23]. Interestingly, hypoxia had much less effect on endothelial sprouting from aortic explants isolated from TP508-injected mice. In fact, explants from TP508-injected mice that were exposed to hypoxia showed sprouting that was equivalent to that seen in normoxic explants from control mice. These results suggest that TP508 systemic administration protects endothelium from the effects of chronic hypoxia.

To determine potential molecular mechanisms of TP508 on endothelial cells that may explain how TP508 systemic administration potentiates VEGF-stimulated endothelial sprouting ex vivo, we performed in vitro studies using primary HCAE cells. A first question was whether the effects of TP508 might be related to TP508-induced production of VEGF. We found that in HCAE cells TP508 did not increase VEGF mRNA expression or VEGF protein production (data not shown). These results are consistent with a previous study showing that direct TP508 treatment of microvessels in culture increased sprout elongation, but did not increase VEGF mRNA expression [37]. Thus, it appears unlikely that in vivo effects of TP508 are mediated through stimulation of VEGF production.

We previously demonstrated that TP508 stimulates NO production in human endothelial cells by different mechanisms than VEGF [30]. Since NO production is required for VEGF-stimulated endothelial cells proliferation [15], migration [13, 32] and tube formation [38], it seemed possible that TP508-stimulated NO production may contribute to enhancement of VEGF-induced endothelial sprouting from aortic explants. Our in vitro studies showed that TP508 and VEGF stimulated NO production in normoxic HCAE cells to nearly the same extent. In hypoxic cells, however, basal levels of NO production and VEGF-stimulated NO production were significantly decreased while TP508 still stimulated NO production by 2- and 3-fold over that of normoxic or hypoxic controls, respectively. Although it is difficult to compare effects observed in cells in culture with events that happen in vivo, these results suggest that production of NO induced by systemically injected TP508 may contribute to the protective effects on hypoxia-induced inhibition of endothelial sprouting from aortic explants. Moreover, previous studies demonstrated that wound healing [39] and angiogenesis is significantly impaired in eNOS-deficient mice and is not improved by administration of VEGF [40], suggesting that NO production by endothelial cells is essential for angiogenesis. If TP508 injected systemically is enhancing angiogenic responses to VEGF and protecting endothelial cells from hypoxia,

one could ask whether this effect is specific for VEGF or whether a TP508 injection might also enhance responses to other angiogenic growth factors.

Preliminary experiments demonstrate that TP508 systemic administration also enhances endothelial sprouting stimulated by FGF (data not shown). That the systemic TP508 effect on endothelial sprouting is not limited to a single growth factor pathway is consistent with the broad range of effects of TP508 that could be mediated by activation of NO signaling in endothelial cells to promote revascularization.

Previous in vitro studies have shown that TP508 stimulates elongation of sprouts from cultured microvessel fragments [37] and that TP508 increases attachment of endothelial cells or chemotaxis [41, 42]. In these in vitro studies, TP508 was added to the medium, where it could have a direct immediate effect on the cells [37], or was attached to the plates or inserts where it could interact with integrins expressed on endothelial cells through an RGD domain in the peptide to stimulate cell attachment and haptotactic migration [41]. However, in our current in vivo studies, TP508 was injected into animals 24 h before aortic fragments were isolated and placed in culture. Thus, the effects of TP508 occurred during the period in which TP508 was in contact with the cells in the animal and persisted through isolation and culture without additional TP508 treatment. Thus, our studies indicate that TP508 induces changes in the endothelial cells that persist after isolation to provide longer-term protection from hypoxia and increased responses to angiogenic factors.

These results are consistent with the hypothesis that TP508 represents a natural breakdown product of thrombin that is released from fibrin clots into circulation to stimulate tissue revascularization and repair [43, 44]. Studies are currently underway in our laboratory to identify the molecular mechanisms by which TP508 potentiates VEGF-stimulated intracellular signaling and endothelial cell functions in HCAE cells.

In summary, these results demonstrate for the first time that a single TP508 intravenous injection promotes angiogenic sprouting and potentiates VEGF-stimulated endothelial sprouting from aortic explants cultured under normoxic conditions. Exposure of aortic explants to hypoxia leads to inhibition of basal and VEGF-stimulated endothelial sprouting. Importantly, TP508 injection protects endothelial cells from the hypoxia-induced attenuation of endothelial sprouting. These results suggest a potential benefit of using a combination of systemic TP508 and local VEGF for treatment of chronic wounds and as a therapy for revascularization of ischemic tissues.

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