Effect of Mini-tyrosyl-tRNA Synthetase on
Ischemic Angiogenesis, Leukocyte Recruitment
and Vascular Permeability

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Short title: Vascular actions of mini-tyrosyl-tRNA synthetase

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ABSTRACT

Mini-tyrosyl-tRNA synthetase (mini-TyrRS), the N-terminal domain of tyrosyl-tRNA synthetase, is a recently identified protein released by endothelial cells that exhibits angiogenic and leukocyte chemo-attractant, ELR-motif-dependent activities in vitro. We sought to determine whether exogenous mini-TyrRS exerts these and other cytokine-like actions in physiological and pathological settings in vivo. High-dose mini-TyrRS (600 ug/kg/day) augmented while low-dose (3 ug/kg/day), unexpectedly, inhibited angiogenesis in the ischemic mouse ear. Enhanced angiogenesis was associated with increased CD45- and CD4-positive leukocyte accumulation. Mini-TyrRS also had biphasic actions on both basal and mustard oil-evoked and VEGF-evoked leakage of Evan’s blue dye-albumen in non-ischemic ear and in endothelial cell monolayers, ie, low-dose inhibited and high-dose augmented leakage. Mutation of the ELR motif of mini-TyrRS abolished the above activities. Mini-TyrRS was reduced (immunoblot) in extracts of ischemic calf muscle and in thoracic aorta explants exposed to hypoxia or VEGF. Inhibition of VEGF with a soluble Flt1 “trap” protein abolished this hypoxic-induced reduction in mini-TyrRS in aorta explants. These data show that mini-TyrRS has dose-dependent biphasic effects on ischemic angiogenesis and vascular permeability in vivo, ie, anti-angiogenic and anti-permeability activities at low concentration and pro-angiogenic, pro-permeability activities at high concentrations.

Key Words: angiogenesis • Mini tyrosyl-tRNA synthetase • hypoxia • ischemia • vascular endothelial growth factor
INTRODUCTION

Aminoacyl-tRNA synthetases, which catalyze the aminoacylation of tRNA molecules, are essential for encoding genetic information during translation(22, 32-35). In higher eukaryotes, aminoacyl-tRNA synthetases associate with other polypeptides to form supra-molecular multi-enzyme complexes. The eukaryotic tRNA synthetases consists of a core enzyme, which is closely related to the prokaryotic counterpart of the tRNA synthetase, and an additional domain that is appended to the amino-terminal or carboxyl-terminal end of the core enzyme. Human tyrosyl-tRNA synthetase (TyrRS), for example, has a carboxyl-terminal domain that is not part of the prokaryotic and lower eukaryotic TyrRS molecules. The synthetase also has an N-terminal domain that is cleaved by several endogenous enzymes, yielding “mini-tyrosyl tRNA synthetase” (mini-TyrRS).

Mini-TyrRS has recently been shown to possess cytokine-like actions, leading to its inclusion in a growing family of aminoacyl tRNA synthetase “AARS” multifunction cytokine-like proteins and peptides(22, 32-35). Mini-TyrRS stimulates neutrophil activation and chemotaxis in vitro, and is angiogenic in endothelial cell cultures and in chick chorioallantoic membrane (CAM) and mouse matrigel implants(22, 32-35). Like CXC-chemokines such as IL-8, mini-TyRS has an ELR motif (Glu-Leu-Arg) that confers its chemokine and angiogenic activities. Mutation of this motif inhibits binding and abolishes stimulation of leukocytes and induction of angiogenesis. Monocytes/macrophages, T-lymphocytes, and endothelial progenitor cells are important in angiogenesis, where they are recruited into and around new capillary sprouts and
secrete growth factors and cytokines that promote endothelial cell proliferation and migration (9, 13).

Despite these intriguing in vitro actions, no studies have examined the mini-TyrRS in physiological or pathological settings in vivo. Therefore, the purpose of this study was to determine if exogenous mini-TyrRS augments angiogenesis and leukocyte recruitment in ischemic tissue in vivo. We also investigated it for other key actions exhibited by angiogenic factors, namely, induction of vasodilation and increased vascular permeability. Our findings show that mini-TyrRS augments angiogenesis and leukocyte adhesion in a mouse ear model of ischemic angiogenesis, and increases permeability but lacks vasoactive actions. Intriguingly, at low concentrations mini-TyrRS had opposite, i.e. angiostatic activity, and reduced baseline and evoked increases in permeability. These novel biphasic actions of mini-TyrRS have potential therapeutic and physiological implications.

**METHODS**

**Reagents.** Rabbit anti-human mini-TyrRS antibody and human recombinant mini-TyrRS were from aTyr Pharma, Inc (La Jolla, CA). mFlt-trap (soluble VEGF-A receptor decoy) was kindly provided by Napoleon Ferrara and Stuart Bunting (Genentech). Bovine coronary venular endothelial cells were a gift from Cynthia Meininger, Texas A&M University. Four-to-five month-old mice were used in ear artery ligation (C57BL/6) and permeability models (sv129); a total of 285 animals were used to obtain the in vivo data shown in the figures. Procedures were performed aseptically and approved by the IACUC of the University of North Carolina at Chapel Hill.
Unilateral ear artery ligation (Fig 1). 1-2mm incisions at the base of the pinna were made overlying the central and peripheral ear artery trunks. Each artery was transected between two 7.0 ligatures placed 1mm apart(1). The central ear artery was ligated distal to its lateral branch to prevent ear necrosis. 20ul of phosphate buffered solution (PBS) containing mini-TyrRS at 4 doses, mutant mini-TyrRS or PBS (vehicle) was injected subcutaneously every 12h.

Laser Doppler perfusion imaging (Fig 1). Animals were anesthetized with 1.125% isoflurane supplemented with 2:3 oxygen:air. Perfusion was obtained before ligation, immediately after, and on 1,3,5 and 7 days using a scanning laser-Doppler perfusion imager (LDI2-IR, Moor Instruments) modified for high resolution(10). Regions of interest (ROIs) were drawn by an investigator blind to drug treatment using Moor software to delineate a region between the ear margin and a circle extending from a line drawn to connect the two pinna notches. Unless otherwise indicated, perfusion was measured at 38°C rectal temperature to reduce vasomotor tone.

Vascular albumen leakage. Albumen leakage (“permeability”) was measured with Evans blue dye (T1824)(18) in the ear or dorsolateral back skin. Mini-TyrRS, mutant mini-TyrRS or PBS was injected (10ul, 32ga needle here and elsewhere) subcutaneously into the ear dorsum at the base of the pinna. Thirty minutes was observed to allow absorption of the volume and resolution of any effects on local interstitial fluid pressure. Then, 25ul T1824 (30mg/kg) was administered via jugular vein.
Immediately afterwards, allyl isothiocyanate (the active ingredient in mustard oil; Sigma; diluted with mineral oil to 5% (v/v)) or mineral oil (control) was applied topically (5ul) to the dorsal and ventral surfaces of both ears with a cotton-tip applicator. Thirty minutes later the vasculature was perfusion-fixed (1% paraformaldehyde (PFA) in 50 mM citrate buffer, pH 3.5) for 1min at 120mmHg. Ears were removed, dried at 55°C for 24h and weighed. Vascular leakage was indicated as T1824 content extracted by incubation in 1mL formamide for 48h at 55°C, and measured with a spectrophotometer at 610 nm against a standard curve(31).

VEGF-induced permeability was examined in the shaved back skin. 20ul PBS containing mini-TyrRS or PBS alone was injected subcutaneously. 30min later T1824 was injected IV as above, followed by VEGF-A\textsuperscript{165} (100ng in 20ul PBS; R&D Systems) or PBS injected subcutaneously at the same location. Thirty minutes later a skin circle, identified with a stereomicroscope circumscribing the extent of blue dye, was excised and T1824 content was determined as above.

**Bovine coronary venule endothelial cell (BCVEC) culture and monolayer permeability.** BCVECs (passage 10-15) were seeded onto culture dishes or onto 0.4μm transwell inserts (Corning) (3x10\textsuperscript{5} cells/insert), both pre-coated with 1.5% gelatin, and maintained in Dulbecco's modified Eagle’s medium (DMEM) with 20% fetal bovine serum (FBS) at 10% CO\textsubscript{2} until a tight confluent monolayer was achieved. Cells were then pre-treated with mini-TyrRS for 10 minutes, followed by 100ng/ml VEGF for 30 minutes. Monolayers were then treated with Evans blue-bovine serum albumin complex (0.67g/l and 40g/l) in HEPES-buffered saline for 30min. Evans blue-albumin in the
lower well was measured at 610nm absorbance. Trans-endothelial albumin flux is expressed as percent clearance of albumin, compared with untreated controls.

**Thoracic aorta explants.** Rat thoracic aortae were isolated and maintained in serum-free medium composed of DMEM/F12, 10mg/l insulin, 5ug/l selenium and 5.5mg/l transferrin in 21% or 1% O₂. After exposure to VEGF for 4 days, samples were frozen in liquid nitrogen for immunoblot assay.

**Immunohistochemistry.** For capillary density, ears were perfusion-fixed with 4% PFA in PBS (pH 7.4) at 120mmHg and then post-fixed in 4% PFA for 24h and embedded in paraffin. Eight micron thick sections located 5500um from the distal tip of the pinna were quantified for capillary density after staining for CD31 (sc-1506, 1:50, Santa Cruz), followed by Cy3-conjugated secondary antibody (1:600). Vessels were imaged in 8 different fields (200X magnification) that covered the entire ear (cartilage, skin surface and hair follicles with auto-fluorescence were excluded in digital images). Capillary density was derived from mean intensity of CD31 immunofluorescence (Image-J). T-cells and leukocytes were stained in adjacent sections with rat anti-mouse CD4 antibody (1:50, sc-13573, Santa Cruz) and CD45 antibody (1:200, 30-F11, BD Pharmingen) respectively, followed with Cy3-conjugated secondary antibody (1:400-600). CD4 and CD45 positive cells were counted for the entire ear cross-section at 400X magnification.
Immunoblot. Tissues were powdered in a liquid nitrogen-cooled pulverizer. Tissues and cultured cells were lysed in 1.5% triton-X100 lysis buffer containing protease inhibitors (30µg/ml aprotinin, 1mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin and 1µg/ml pepstatin) and phosphatase inhibitors (1mM sodium-orthovanadate, 2.5mM sodium-pyrophosphate). Samples were electrophoresed through 10% SDS-polyacrylamide and transferred to nitrocellulose membranes. Membranes were probed with antibodies against mini-TyrRS (1:1000 dilution) and tubulin (1:5000, ab6160, Abcam) followed by Alexa Fluor-680 (Molecular Probes) or IRDye 800 (Rockland) conjugated secondary antibodies at 1:5000. Membranes were scanned and analyzed with the Odyssey system (LI-COR Biosciences).

Statistics. Data are given as means ± SEM. Differences were subjected to unpaired t-tests (2-tailed) or ANOVA followed by Bonferroni tests for multiple comparisons (2-tailed). 

$P < 0.05$ was considered significant.

RESULTS

Mini-TyrRS has biphasic effects on ischemic angiogenesis

Previous studies have reported that mini-TyrRS induced angiogenesis in cultured endothelial cell, CAM and mouse matrigel assays(22, 32-35). To determine if mini-TyrRS has angiogenic activity in vivo in ischemia, we examined its effects in the mouse ear made ischemic by ligation of the peripheral and central ear arteries, leaving the proximal-lateral branch of the central artery intact (Fig 1). This is the first description of a mouse ear model for quantitative measure of perfusion, angiogenesis and
permeability. We developed it because of its ready access for these measures, plus its amenability to local administration of agents. In the PBS control group, perfusion declined 50% immediately after ligation, followed by recovery within 3-5 days mediated by angiogenesis and growth of collaterals between the above arterial trees (Fig 1A). Local subcutaneous injection (20ul, twice daily every 12h) of 3 ug/kg/day mini-TyrRS into the base of the ear inhibited —while 600 ug/kg/day augmented— recovery of perfusion. Doses of 0.05 and 30 ug/kg/day had no effect (n=8 mice/dose, data not shown), underscoring the biphasic activity. Doses higher than 600 ug/kg/day were not tested for reasons of concentration and cost. Mutant mini-TyrRS (ELR mutated to EYR) had no effect.

We next examined the effective doses in the figure 1A experiment for effect on capillary density measured 7 days after ligation. The biphasic effect of mini-TyrRS on recovery of ear perfusion was accompanied by similar changes in capillary density (Fig 1B). Capillary density, which increased in ischemia as expected, was inhibited by 3 ug/kg/day and augmented by 600 ug/kg/day mini-TyrRS, whereas mutant mini-TyrRS had no effect. This biphasic activity is different from *in vitro* where only dose-dependent stimulation of angiogenesis occurs. Body weight did not differ between PBS or drug groups at any time point. In addition, daily subcutaneous injection of mini-TyrRS in the ear was not accompanied by erythema or edema.

**Mini-TyrRS increases leukocyte accumulation in ischemia**

Monocytes/macrophages and T-lymphocytes are involved in ischemic angiogenesis *in vivo* (9, 17). Furthermore, mini-TyrRS stimulates monocyte adhesion and
transmigration \textit{in vitro}(13, 19, 22). We therefore examined whether mini-TyrRS affects leukocyte density in cross-sections of the ear adjacent to those used for determining capillary density in figure 1B. As expected, CD45$^+$ and CD4$^+$ cells increased after ligation (Fig 2). At low-dose mini-TyrRS which inhibits angiogenesis, no effect on leukocyte density was observed. However, high-dose mini-TyrRS caused a further increase of both cell types, whereas mutant mini-TyrRS was without effect.

**Mini-TyrRS has biphasic effects on baseline and evoked increase in permeability**

Increased permeability of the endothelium is an important step in the initial phase of sprouting angiogenesis, and several angiogenic factors, eg VEGF, regulate angiogenesis in part through alterations in permeability(2, 7, 9, 20, 25). We thus examined whether mini-TyrRS modulates leakage ("permeability"), using extravasation of Evans blue-conjugated albumen in normal, non-ligated ears. Similar to its biphasic effects on angiogenesis (Fig 1), mini-TyrRS also had biphasic effects on permeability (Figs 3 and 4). Low-dose mini-TyrRS (30ug/kg) reduced baseline leakage by 50%, while high-dose (600ug/kg) increased leakage greater than 2-fold (Fig 3A); mutant mini-TyrRS had no effect. We also tested mini-TyrRS on mustard oil-induced increase in permeability. Mini-TyrRS caused dose-dependent inhibition of induced leakage at low doses, with maximal inhibition at 3ug/kg, whereas 600ug/kg slightly augmented induced permeability but not significantly (Fig 3B). Mutant mini-TyrRS had no effect, suggesting—like its actions on angiogenesis, leukocyte accumulation and baseline permeability—that the ELR motif is required for mini-TyrRS’s modulation of mineral oil-induced permeability. Similar results were obtained with VEGF-induced leakage (Fig 4A). The
biphasic action of mini-TyrRS on VEGF-induced leakage was confirmed in endothelial cell monolayers (Fig 4B).

**Mini-TyrRS lacks vasoactive actions**

Because some angiogenic factors such as VEGF and bFGF exhibit vasodilator and vasoconstrictor activity, respectively(9), and since such activity of mini-TyrRS could alter perfusion in the ear ligation and permeability in the Evans blue experiments, we evaluated the effect of mini-TyrRS on perfusion of the normal (non-ligated) mouse ear. The following protocols (conducted in separate animals) reflect two requirements in these *in vivo* experiments: First, ten minutes were required to obtain the laser scanning Doppler perfusion measurement. Second, 20-30 minutes were allowed after a local injection (20 ul for all agents tested) to permit any elevation of interstitial pressure to subside.

In the first experiment, rectal temperature was lowered to 35°C to increase vascular tone. Baseline perfusion was then obtained, followed by injection of mini-TyrRS or PBS into the base of the ear. Rectal temperature was then raised to 37.5°C over 10 minutes to cause warming-induced vasodilation, and perfusion was obtained again. Mini-TyrRS had no effect on warming-induced dilation (Fig 5A). In a second experiment, baseline perfusion was obtained at 35°C, mini-TyrRS or PBS was then injected, 30 minutes later the vasodilator papaverine was injected in the same location, and perfusion was obtained again 30 minutes later. Although there was a slight suggestion that mini-TyrRS might modestly reduce the dilation (Fig 5B), the peptide had no significant statistical effect on papaverine-mediated dilation. If mini-TyrRS has vasoconstrictor or
vasodilator actions, dilator response to the superimposed warming period in the first experiment or to papavarine in the second experiment should have been altered. In a third experiment to investigate possible progressive vasoactive actions with repeated exposure, mini-TyrRS was administered locally on 6 consecutive days. Ear perfusion was measured at 36.5°C 24h after each administration and just before repeat-dosing. Mini-TyrRS had no effect on perfusion on day-6 (Fig 5C) nor at any of the 5 earlier days (data not shown). Absence of vasoactive effects in these experiments is consistent with absence of erythema noted at any times or dosages, either immediately after or 24 hours after mini-TyrRS administration. Edema was not evident at the 600 ug/kg dosages in any of the experiments, even though this dose increased baseline permeability (Fig 3A). The may reflect low interstitial compliance and/or efficient lymphatic drainage in the ear.

Ischemia, hypoxia and VEGF reduce mini-TyrRS in calf and isolated aorta

Angiogenesis in response to tissue hypoxia and ischemia is achieved through upregulation of angiogenic factors such as VEGF, which—in turn or through other mechanisms—downregulate angiostatic factors(9, 30). We thus reasoned that if endogenous mini-TyrRS normally exerts angiostatic actions at low concentrations, as suggested by the above findings showing that low-dose mini-TyrRS inhibited recovery of flow, angiogenesis and permeability, then tissue levels of mini-TyrRS might be regulated negatively in ischemia and in response to VEGF. We used mouse hindlimb ischemia to examine this question because it is a widely used model of neovascularization and because it is difficult to extract sufficient protein of high quality
from the cartilaginous and fibrous ear. In addition, this model allowed measurement of mini-TyrRS by immunoblot in muscle that experiences ischemia (gastrocnemius) versus little or no ischemia (adductor) after femoral artery ligation(10, 18). Tissue samples were harvested on days 2, 5 and 10 after ligation of the femoral artery, as detailed elsewhere(7). Mini-TyrRS decreased in the gastrocnemius but not adductor of the ligated leg, when compared to the gastrocnemius from sham animals (no surgery) or from the contralateral non-ligated leg (Fig 6A, B). The specificity of the mini-TyrRS band identified with rabbit anti-human antibody was verified using a second goat anti-rabbit antibody we raised against a different epitope of mini-TyrRS. To test possible involvement of hypoxia and VEGF in reduced mini-TyrRS, we examined rat thoracic aorta maintained in organ culture. Four days of exposure to VEGF or hypoxia (1% O₂) caused similar reduction of mini-TyrRS (Fig 6 C, D). Moreover, hypoxic reduction was abolished by VEGF neutralizing trap. These data suggest that hypoxic induction of VEGF may mediate reduced mini-TyrRS in ischemic tissue.

DISCUSSION

This is the first characterization of the effects of exogenous mini-TyrRS on ischemic angiogenesis, leukocyte trafficking, permeability and vasoactivity in vivo. We confirm several properties previously observed in vitro, identify novel biphasic actions on angiogenesis and permeability, and find evidence suggesting that tissue levels of mini-TyrRS may be regulated by hypoxia.

In the mouse ear model of ischemia, low-dose mini-TyrRS (3 ug/kg/day) inhibited while high-dose (600 ug/kg/day) augmented angiogenesis. Intermediate doses of 0.05
and 30 ug/kg/day had no effect. Mutant mini-TyrRS had no effect, confirming the requirement of an intact ELR. The angiostatic-like action of low-dose mini-TyrRS was not observed in previous in vitro, matrigel and CAM implant studies where only angiogenic actions were observed. For example, mini-TyrRS was angiogenic at 2.4-24 ug/ml (60-600 nmol/L) in matrigel implants and induced migration of cultured endothelial cells at 2 ug/ml (50 nmol/L)(33). These differences could arise for several reasons, including the context of ischemia in our study, inherent differences in conditions in vivo, in vitro and “in matrigel”, and because the concentrations used previously are undoubtedly higher than achieved in our low-dose groups where dilution and degradation over time would be considerable. For example, assuming mini-TyrRS injected locally into the ear distributes into the extracellular space and that degradation reduces the concentration by 10 fold (actual degradation is likely to be a least one order of magnitude more than this), our low- and high-dose regimens would achieve average extracellular concentrations of 0.006 ug/ml and 1.2 ug/ml. Thus, although concentrations in the ear’s extracellular fluid would clearly be higher for some duration after injection, it is likely that time-averaged levels achieved in our low-dose groups were significantly lower that in previous studies.

Recruitment of leukocytes and endothelial progenitor cells (EPCs) contributes importantly to angiogenesis in ischemia, inflammation and tumor growth(2, 9, 13, 20). These cells exhibit heterogeneous phenotypes, expressing markers for macrophages, T-cells, SMCs, fibroblasts, pericytes and EPCs, and secrete growth factors and cytokines which act directly or indirectly to augment endothelial cell migration, proliferation and capillary sprouting. CD4-positive T-lymphocytes play an important role
in angiogenesis by secreting angiogenic growth factors such as VEGF(14) and bFGF(4). In the present study, high-dose mini-TyrRS further increased leukocyte (CD45-positive) accumulation in the ischemic ear by ~2.5 fold compared to PBS control and CD4-positive cells by ~10 fold, while low-dose mini-TyrRS had no effect. This action may contribute to the angiogenic effect of mini-TyrRS.

Besides the novel biphasic effects of mini-TyrRS on angiogenesis, mini-TyrRS also had biphasic effects on both basal and evoked permeability. In most vascular beds, including the skin, permeability to plasma proteins and smaller molecules is normally low. Ischemia, inflammation and tumor growth are accompanied by increased vascular permeability which is an important early step in angiogenesis in these conditions(2, 9, 27). The resulting leakage of plasma proteins and other circulating macromolecules helps to convert the normally anti-angiogenic stroma into a pro-angiogenic provisional stroma(9, 11). Many angiogenic factors such as VEGF(16), bFGF(7), interleukin-8(25) and thrombin(16) increase endothelial permeability(2). On the other hand, antagonism of increased permeability reduces angiogenesis(5, 29, 30). For example, the angiostatic proteins angiostatin(28), caveolin-1(5) and TNP-470(26) reduce evoked increases in permeability. However to our knowledge, no endogenous angiostatic factor has been reported to reduce basal permeability like that observed in the present study for mini-TyrRS. On the other hand, high-dose mini-TyrRS increased basal permeability. We also found that mini-TyrRS at low doses caused dose-dependent inhibition of evoked leakage by mustard oil and VEGF, while high-dose mini-TyrRS tended to augment evoked leakage. This positive regulatory effect of mini-TyrRS on basal and evoked permeability may contribute to the angiogenic action of mini-TyrRS. Although the
mechanisms underlying the biphasic effect await future studies, estrogen has similar biphasic effects on permeability in ECs in vitro (36).

The specificity of our findings regarding angiogenesis, leukocyte accumulation and increased permeability are supported by their dependence on an intact ELR (Glu-Leu-Arg) motif, ie, mini-TyrRS with this motif mutated had no effects. This motif is required for binding, neutrophil activation/adhesion and angiogenesis induced by mini-TyrRS in vitro and other CXC ELR-containing chemokines such as Interleukin-8 (15, 16, 22, 32, 33, 35). However, the ELR motif need not be angiogenic. For example, the ELR motif-containing chemokine GRO-β has anti-angiogenic properties (8), and members of the CXC subfamily, induced by interferons, lack the ELR motif but are potent inhibitors of angiogenesis (19). In addition—and of potential relevance to our findings—, 22-26-amino acid peptides derived from pro-angiogenic ELR-containing CXC chemokines exhibit potent anti-proliferative and anti-migratory activity in vitro (19). The mini-TyrRS receptor has not yet been identified, nor is it known if mini-TyrRS binds to different receptors at low versus high concentrations. Multiple receptors and/or processing of the protein after export from endothelial and other vascular cells (15, 22, 35) could underlie the biphasic properties we observed. For example, the AARS cytokines mini- and T2-TrpRS, thought to be derived from proteolysis and/or alternative splicing of TrpRS, inhibit VEGF signaling and are angiostatic (21, 22, 34, 35). It has recently been reported in endothelial cells that mini-TyrRS release is induced by TNFα, undergoes binding, and induces phosphorylation of Src, Akt, ERK and VEGF-receptor 2 which is required for tube formation (15). These factors are also involved in signaling angiogenesis, angiostatic activity, leukocyte adhesion and changes in vascular permeability (2, 9, 20,
23, 30), and thus could be involved in the biphasic action of mini-TyrRS on these processes identified in herein. Knowledge of the signaling pathways activated at low versus high concentrations of mini-TyrRS in vivo or in intact vessel organ culture preparations will be required to understand the basis for the biphasic activities identified in this study.

“Tissue hypoxia in ischemia and tumor growth induces many of the steps involved in angiogenesis, eg, increased permeability, inflammation, endothelial cell proliferation and migration, and matrix degradation(2, 3, 6, 9, 12, 20, 23, 24). A number of the proteins that mediate these processes, such as eNOS, VEGF, angiopoietin-2, AKT and bFGF are regulated by hypoxia. Mini-TyrRS levels detected by immunoblot of tissue lysates were significantly reduced in gastrocnemius muscle when examined at 5 and 10 days after ligation of the femoral artery. Similar reductions were also observed in thoracic aorta explants exposed to either hypoxia or VEGF. Moreover, in the latter model a VEGF-trap blocking protein abolished reduction in mini-TyrRS during hypoxia. These findings in explants suggest that the reduction of mini-TyrRS in calf muscle after femoral ligation in vivo may be mediated by hypoxia and VEGF. This supports our hypothesis from the findings in figure 1 that the physiological effects of low concentrations of mini-TyrRS are angiostatic. It is possible that the decline in mini-TyrRS detected in tissue extracts of calf and aorta explants reflects a regulated reduction in cleavage of the protein from full-length TyrRS to cause withdrawal of its angiostatic effect produced at low concentration (Fig 1). On the other hand, it has recently been shown that mini-TyrRS is released by stimulation of endothelial cells with TNFα in vitro(15). Thus, it is also possible that export from endothelial cells (and possibly other cell types) in
response to ischemia, hypoxia and VEGF (conditions tested herein; however VEGF did not induce release of mini-TyrRS \textit{in vitro}(15)) could have reduced intracellular levels. This plus proteolysis of any released mini-TyrRS may have resulted in lower levels detected in tissue extracts. Additional studies and development of assays to measure released mini-TyrRS will be required to determine if the decline in mini-TyrRS with the models and stimuli examined herein reflects increased export and achievement of angiogenic concentrations or is due to a regulated reduction in levels to withdraw the angiostatic activity of low mini-TyrRS levels. If evidence for the latter is obtained, the angiogenic and related actions of high-dose mini-TyrRS that we observed may reflect a potential therapeutic use of the protein in ischemia, while the low-dose angiostatic and barrier-increasing actions may reflect physiologic activities as well as a potential therapeutic use to inhibit pathological angiogenesis.” Clearly, more work needs to be done to understand expression/ proteolysis of TyrRS under different physiological conditions.

In summary, we report that low-dose mini-TyrRS inhibits basal and evoked permeability and ischemic angiogenesis. High-dose has opposite effects and, in addition, augments recruitment of CD45-positive and CD4-positive cells in ischemic tissue. To our knowledge, mini-TyrRS is the first factor observed to inhibit angiogenesis at low and stimulate it at high concentrations. We also provide \textit{in vitro} evidence in aorta that mini-TyrRS levels are reduced by VEGF-dependent signaling in hypoxia, suggesting a similar mechanism may underlie its reduction in ischemic tissue \textit{in vivo}. These findings suggest that endogenous mini-TyrRS may serve an angiostatic function
in certain physiological settings. Moreover, administration of it at low versus high levels could provide a therapeutic approach to limit versus augment angiogenesis in certain diseases. Important areas of future study include determining whether stimulation of leukocyte accumulation by mini-TyrRS reflects induction of adhesion molecules on endothelial cells, leukocytes or both, or release of inflammatory cytokines from these or other cell types, and similar questions regarding the biphasic effects of mini-TyrRS on baseline and induced permeability and ischemic angiogenesis. Besides a number of tools that need to be developed, strategies to antagonize endogenous production/cleavage/secretion and block the responsible receptor(s) will be needed to test the potential physiological roles suggested by the findings in this study.
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REFERENCES


FIGURE LEGENDS

Figure 1. Mini-TyrRS has biphasic actions on recovery of blood flow and angiogenesis in a model of ear ischemia. Top: Doppler perfusion was determined for the ear in an anatomically defined region of interest (ROI; see MATERIALS AND METHODS) before and at indicated times after ligation of the central and peripheral ear arteries (“X” in first panel); same mouse (PBS control) used in each panel. Pseudocolor bar spans 0–5,000 perfusion units. A, Perfusion values normalized to non-ligated contralateral ear. Recovery of ear perfusion was inhibited by low- and augmented by high-dose mini-TyrRS (mTyrRS), while “mutant” mini-TyrRS had no effect (“mutant” here and elsewhere is mini-TyrRS with the ELR motif mutated to EYR). Doses of 0.05 and 30 ug/kg/day had no effect (n=8 mice/dose, data not shown), underscoring the biphasic activity. Ear doses are the total daily dose from 2 injections given subcutaneously into the base of ear (“∗” in first panel) 12 hours apart. # p<0.05, ## p<0.01, ANOVA; ** p<0.01, Bonferroni t-test; 2-tailed tests here and other figures. B, Angiogenesis (capillary density, measured at day 7 after ligation) was inhibited by low-dose and augmented by high-dose mini-TyrRS, while mutant mini-TyrRS had no effect compared with PBS. Fluorescence intensity of CD31 antibody was averaged for 8 fields spanning entire ear cross-section (See Supplment Data for representative histological images). #p<0.05, ##p<0.01, ###p<0.001 vs. sham ligation; *p<0.05, **p<0.01 vs. PBS. N=6-9/bar. Values are mean±SEM for “N” number of animals for this and subsequent figures.

Figure 2. High-dose mini-TyrRS augments accumulation of CD45- and CD4- positive cells 7 days after ear artery ligation. Representative 8-micron thick sections of ear
stained with anti-CD45 and anti-CD4 antibodies. CD45-positive cells are the average of 10 high power fields. CD4-positive cells are for entire ear cross-section. *p<0.01 vs. sham ligation, #p< 0.001 vs. PBS. N=6-9/bar.

**Figure 3.** Mini-TyrRS has biphasic actions on baseline and mustard oil (MO) evoked increase in macromolecular leakage in ear. A, mini-TyrRS, alone, at low-dose (sc) reduced and at high-dose augmented leakage, while mutant mini-TyrRS had no effect. B, Low-dose mini-TyrRS inhibited MO-induced ear leakage, while high-dose or mutant mini-TyrRS had no effect. Trans-endothelial albumin flux expressed as percentage of clearance of Evans blue-conjugated bovine serum albumin, compared with untreated controls. $p<0.05, #p<0.01 vs. PBS, *p<0.05, ***p<0.001 vs. MO. N=4-7/bar.

**Figure 4.** Mini-TyrRS has biphasic actions on VEGF evoked increase in macromolecular permeability. A, Low-dose mini-TyrRS (sc) inhibited VEGF-induced leakage in dorsolateral trunk skin. B, Mini-TyrRS had biphasic actions on VEGF-induced leakage in endothelial cell monolayers. Trans-endothelial albumin flux expressed as percentage of clearance of Evans blue-conjugated bovine serum albumin, compared with untreated controls. #p<0.01 vs. PBS, *p<0.05, **p<0.01 vs. VEGF. N=4-7/bar.

**Figure 5.** Mini-TyrRS lacks vasoactive actions. A, Increase in perfusion (Doppler) induced in non-ligated ear by raising rectal temperature from 35°C to 37.5 °C was
unaffected by mini-TyrRS (20ul subcutaneous administration into ear immediately after 35°C measurement, followed by measurement 10 min later at 37.5°C). B, Increase in perfusion induced by papavarine (adductor area sc) was unaffected by mini-TyrRS injected into same site 30 min earlier. C, Baseline (control) ear perfusion was unaffected after 6 days of daily sc mini-TyrRS (daily dose given as two 20ul injections 12 hours apart). N=4-6/bar.

**Figure 6.** Ischemia/hypoxia and VEGF reduce mini-TyrRS levels *in vivo* and *in vitro*. A and B, Western blot analysis of mini-TyrRS in gastrocnemius and adductor of sham-surgery mice, in gastrocnemius and adductor of leg with femoral artery ligation (lig), and in gastrocnemius of the contralateral non-ligated leg at the indicated days (d) after surgery. 20ug (panels A,B) and 30ug (panels C,D) protein per lane; normalized to tubulin. N=4/bar. C and D, Western blot analysis of mini-TyrRS in rat thoracic aorta maintained 4 days in organ culture with 100ng/ml VEGF or 1% oxygen (hypoxia) ± VEGF trap or IgG control (0.2mg/ml). *p<0.05, **p<0.01 vs. sham or normoxia. N=3-4/bar.
Figure 1
Figure 2

CD45 positive cell (per 40x field of view)

- sham ligation
- PBS
- 3 µg/kg mTyrRS
- 600 µg/kg mTyrRS
- 600 µg/kg mutant mTyrRS

CD4 positive cell/ear

- sham ligation
- PBS
- 3 µg/kg mTyrRS
- 600 µg/kg mTyrRS
- 600 µg/kg mutant mTyrRS

*: p < 0.05
#: p < 0.01
Figure 3
Figure 6