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Systemic VEGF inhibition accelerates experimental atherosclerosis and disrupts endothelial homeostasis – implications for cardiovascular safety ☆

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ABSTRACT

Objectives: This study sought to examine the effects and underlying mechanisms of systemic VEGF inhibition in experimental atherosclerosis and aortic endothelial cells.
Background: Pharmacological inhibition of *vascular endothelial growth factor* (VEGF), a major mediator of angiogenesis, has become a widely applied treatment of certain cancers and multiple ocular diseases including *age-related macular degeneration*. However, recent clinical trials raise concern for systemic vascular adverse effects, prompting the Food and Drug Administration to revoke the approval of bevacizumab for metastatic breast cancer.
Methods: Eight-week old apolipoprotein E knockout mice received a high-cholesterol diet (1.25% cholesterol) for 24 weeks and were exposed to a systemic pan-VEGF receptor inhibitor (PTK787/ZK222584, 50 mg/kg/d) or placebo (gavage) for the last 10 weeks. Atherosclerotic lesions were characterized in thoraco-abdominal aortae and aortic arches. Mechanistic analyses were performed in cultured human aortic endothelial cells.
Results: Systemic VEGF inhibition increased atherosclerotic lesions by 33% whereas features of plaque vulnerability (i.e. necrotic core size, fibrous cap thickness) remained unchanged compared with controls. Aortic eNOS expression was decreased (trend). In *human endothelial cells* VEGF inhibition induced a dose-dependent increase in mitochondrial superoxide generation with an uncoupling of eNOS, resulting in reduced NO availability and decreased proliferation.
Conclusion: Systemic VEGF inhibition disrupts endothelial homeostasis and accelerates atherogenesis, suggesting that these events contribute to the clinical cardiovascular adverse events of VEGF-inhibiting therapies. Cardiovascular safety profiles of currently applied anti-angiogenic regimens should be determined to improve patient selection for therapy and allow close monitoring of patients at increased cardiovascular risk.

1. Introduction

Considerable advances in the understanding of the significance of angiogenesis in tumor growth and neovascular ocular diseases have boosted the development and clinical application of anti-angiogenic therapies in the last decade [1–3].

Pharmacological inhibition of vascular endothelial growth factor (VEGF) improved clinical outcomes in different cancer entities [4–6] and revolutionized the treatment of neovascular age-related macular degeneration (AMD), a leading cause of blindness in the developed world [7–10]. In addition, intravitreal anti-VEGF therapy has become the standard of care for macular edema secondary to retinal vein occlusion (RVO) or diabetic retinopathy (diabetic macular edema, DME) [11,12].

However, evidence from recent meta-analyses of randomized controlled trials using anti-angiogenic regimens in cancer (i.e. non-small cell lung cancer, metastatic colorectal cancer, and renal cell carcinoma) accumulates that unanticipated, potentially life-threatening cardiovascular side effects occur upon VEGF inhibition: They include hypertensive emergencies and arterial thrombembolic events encompassing myocardial infarction, cerebrovascular insults, and peripheral or mesenteric ischemia [13–16]. Indeed, in light of significant cardiovascular

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side effects, the Food and Drug Administration (FDA) revoked its approval of bevacizumab (Avastin®) for the first-line treatment of patients with HER2-negative metastatic breast cancer in November 2011 [17]. Despite completed phase III clinical trials the cardiovascular safety profile of VEGF-scavenging based therapies of AMD, RVO and DME patients using either ranibizumab (Lucentis®), bevacizumab (Avastin®) or the recently approved aflibercept (Eylea®) remains insufficiently assessed and thus raises considerable concern [18,19]. All agents enter systemic circulation after intraocular injection, indicating a prolonged *systemic* suppression of VEGF for up to 28 days or longer [2,9,20,21]. The markedly overlapping risk factor profile for the development of AMD and atherosclerotic disease suggests that both diseases tend to occur in the same patient population. Similarly, in RVO and DME patients cardiovascular co-morbidities are most common. Therefore, the cardiovascular safety profile of the exponentially increasing anti-angiogenic therapy regimens for multiple ocular and oncologic diseases needs to be carefully addressed.

Vascular toxicity has been reported irrespective of the mode of VEGF blockade, i.e. both upon treatment with VEGF-trapping antibodies and upon application of receptor tyrosine kinase inhibitors, indicating common underlying mechanisms [22]. Of note, evidence for a beneficial role of VEGF inhibition in vascular biology remains highly controversial [23]. Both systemic and local VEGF gene transfer protected from neointimal growth, a phenomenon that has been reported to be in part NO-dependent [24–26]. Concomitantly, systemic VEGF gene transfer did not enhance experimental atherosclerosis [27]. A single intraperitoneal application of VEGF, however, did increase atherosclerosis in different animal models [28]. Based on human autopsy samples and experimental analyses, neoangiogenesis-dependent intra-plaque hemorrhage and subsequent macrophage infiltration through vasa vasorum has been postulated to promote progression and instability of coronary atheromata [29,30]. However, no evidence for increased atherosclerosis upon systemic or intramyocardial VEGF application was reported in clinical trials [31–33]. A VEGF polymorphism going along with higher VEGF expression was even associated with athero-protective effects [34].

In light of the increasing number of patients receiving anti-angiogenic therapies, this study sought to elucidate the effects of VEGF inhibition on the progression of atherosclerotic lesions in a proof-of-principle study. We therefore used an approach in which mice with existing atherosclerotic lesions were treated systemically with a pan-VEGF receptor inhibitor, *mimicking* the situation of elderly patients receiving anti-angiogenic therapy.

2. Methods

2.1. Animals and diets

Mice were housed in cages with access to food and water in a temperature-controlled room with a 12-hour dark/light cycle. All experiments and animal care procedures were approved by the local veterinary authorities and carried in accordance with our institutional guidelines. 8-week-old male C57BL/6 apolipoprotein E knockout (*ApoE*$^{-/-}$) mice (Jackson Laboratories) were fed a 1.25 % (wt/wt) cholesterol diet (Research Diets) for 24 weeks, and were exposed to a systemic pan-VEGF-receptor inhibitor (PTK787/ZK222584, 50 mg/kg/d, Novartis, [35] in the following termed "PTK787") or placebo by gavage for the last 10 weeks of treatment.

2.2. Assessment of atherosclerosis

For *en face* analyses thoraco-abdominal aortae were excised and opened longitudinally. Atherosclerotic plaques were visualized by fat staining, and analyzed *en face* as described [36,37]. Complementary analyses of plaque size and composition were performed in serial longitudinal cryosections of aortic arches as described [38]. Fibrous cap thickness and necrotic core size were assessed by Elastic van Gieson's (EvG) staining [39].

2.3. Immunohistochemistry and immunofluorescence

Cryosections were blocked and stained using the following antibodies: rat anti-CD68 (Serotec), rat anti-CD31 (BD Pharmingen), rabbit anti-von Willebrand Factor (vWF, Dako), rabbit anti-endothelial nitric oxide synthase (eNOS, Santa Cruz Biotechnology), and mouse anti-proliferating cell nuclear antigen (PCNA, Dako). Collagen was visualized using EvG staining.

2.4. Blood analyses

Mice were fasted over night before blood was drawn prior to harvesting. Whole blood was assessed for complete blood counts. For plasma analyses citrate plasma was separated from corpuscular elements by centrifugation at 4 °C immediately after blood was drawn and stored at −80 °C until analysis. Plasma values of interferon gamma (IFNγ), interleukin 10 (IL-10), IL-1ß, IL-6, and tumor necrosis factor alpha (TNFα) were determined using multiplex array systems (BD Medical Supplies).

2.5. Functional in vitro analyses

Human aortic endothelial cells (HAEC, Cambrex) from passage five to eight were grown to confluence at 5% CO_2 and 37 °C. Functional *in vitro* assays were carried out 24 h after administration of the indicated concentrations of drug or vehicle (DMSO, 0.1% vol/vol). Proliferation was assessed by bromodeoxyuridine (BrdU, Roche Diagnostics) incorporation over 18 h, beginning 6 h after anti-angiogenic treatment. Real-time nitric oxide (NO) generation was investigated through fluorogenic diaminofluoresceindiacetate (DAF2-DA, Sigma-Aldrich) transformation. Intracellular superoxide generation was analyzed using election spin resonance (ESR) spectroscopy as described using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetra-methylpyrrolidine (CMH, Noxygen) [40]. Mitochondrial superoxide generation was investigated through the oxidation and fluorogenic nucleic acid binding of a mitochondrial- and superoxide-specific probe (MitoSOX™, Invitrogen). Cytotoxicity was assessed by lactate dehydrogenase (LDH) release into the supernatant using a commercially available Cytotoxicity Detection kit (Roche Diagnostics). NADPH oxidase activity was quantified through colorigenic conversion of extracted NADPH using an NADPH/NADP Assay kit (Abcam).

2.6. Expression analyses

For in-cell protein analyses, HAECs were fixed in 96-well plates, permeabilized and incubated with rabbit anti-eNOS (Santa Cruz). Specific signals were detected using near-infrared fluorophore-labeled species-specific secondary antisera (Licor Biosciences) and quantified in a LI-COR Odyssey® Scanner. Signals were normalized to DNA content detected by the far-red fluorescent DNA dye DRAQ5™ (Biostatus).

To assess uncoupling of the functional eNOS homodimer, HAEC lysates were separated by electrophoresis under non-denaturing conditions at 4 °C, blotted, and incubated with rabbit anti-eNOS (Santa Cruz). The specific signal was detected using species-specific secondary antisera.

2.7. Statistical analyses

Metric variables were assessed for distribution using Kolmogorov–Smirnov tests; variances of different groups were compared by F tests. Different groups were compared using unpaired Student's *t* tests, Mann–Whitney *U* tests, one-way ANOVA or Kruskal–Wallis tests where appropriate, followed by Dunn's Multiple Comparison tests where applicable. Results are displayed as interquartile ranges ± minimal/maximal values or mean ± SEM, if not indicated differently. At least three independent experiments in triplicates were performed. Significance was accepted at $p < 0.05$, *p* values are two-sided. Analyses were done using Graphpad Prism version 5.0d 2010.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

3. Results

3.1. Systemic VEGF-R inhibition increases experimental atherosclerosis

PTK787 treatment of *ApoE*$^{-/-}$ mice resulted in a significant increase in atherosclerosis as assessed both in thoraco-abdominal aortae *en face* and in longitudinal cross sections of the aortic arch (Fig. 1A, B). Moreover, we observed a trend towards an enhancement of plaque-resident CD68-positive macrophages after VEGF-R inhibition, indicating increased vascular inflammation (Fig. 1C). Plasma levels of circulating inflammatory cytokines, including IFNγ, IL-10, IL-1ß, IL-6, and TNFα did not differ between the intervention and the control group (Table 1A), suggesting a local vascular effect. Concomitantly, no differences in the white blood cell counts and differentials were observed (Table 1B).

3.2. VEGF-R inhibition does not affect features of plaque stability or the number of plaque-resident endothelial cells

To assess putative effects on plaque architecture we compared necrotic core diameters and areas as well as fibrous cap thicknesses. In collagen stainings no differences were observed (Fig. 2). Signs of decreased intra-plaque neoangiogenesis after VEGF-R inhibition were investigated

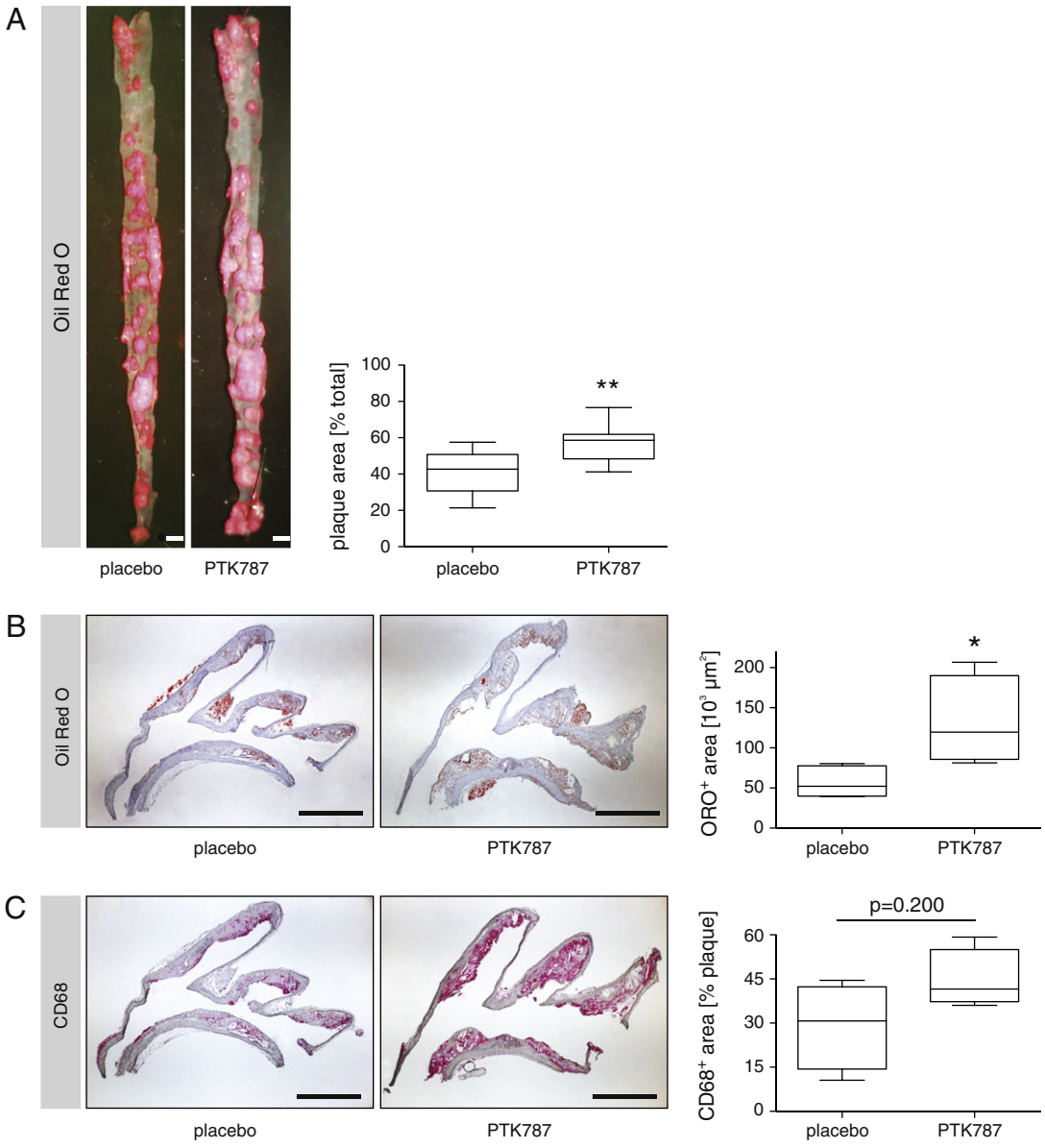

Fig. 1. *Systemic VEGF-R inhibition increases atherosclerotic plaque burden*: 8-week-old male *ApoE*$^{-/-}$ mice on a high cholesterol diet (1.25% w/w) were exposed to the pan-VEGF receptor [VEGF-R] inhibitor PTK787 (50 mg/kg/d, gavage) or placebo for 10 weeks before aortae were explanted and atherosclerotic lesions assessed. (A) Thoraco-abdominal aortae *en face*, oil red o staining (purple), scale bars = 1 mm. $n = 10$ per group. (B, C) Photomicrographs of aortic arches, longitudinal sections, oil red o- [ORO-] (B) and CD68- (C) staining (both purple), scale bars = 1 mm, $n = 5$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$; graphs show interquartile ranges, whiskers indicate minima and maxima.

by tracking endothelial-specific markers in atherosclerotic lesions. Signals for CD31 and vWF in the atherosclerotic aortic wall did not differ between the intervention and the control group (Fig. 3).

3.3. Inhibition of VEGF-R decreases proliferating cells within atherosclerotic lesions

Local functional effects in the aortic wall were characterized by assessing cellular proliferation and the expression of eNOS. The number of proliferating cells as assessed by PCNA stainings in the aortic wall was decreased upon VEGF-R inhibition (Fig. 4A). eNOS-specific signals tended to be reduced in the endothelial layer of intervention group compared with controls, however without reaching significance (Fig. 4B).

3.4. In human aortic endothelial cells VEGF-R inhibition diminishes eNOS expression and function in a dose-dependent fashion

To gain further insight into the mechanisms of VEGF-R inhibition in the vascular wall, dose-response experiments were performed in human aortic endothelial cells. Following up on the trend towards reduced eNOS expression *in vivo*, we observed a dose-dependent decrease of eNOS in human aortic endothelial cells in response to PTK787 (Fig. 5A). Moreover, enzymatic function of eNOS was diminished upon PTK787 treatment in a dose-dependent manner as assessed by uncoupling experiments (Fig. 5B). Accordingly, endothelial nitric oxide release was impaired when inhibiting VEGF receptors in human aortic endothelial cells (Fig. 5C). Given the delicate balance of endothelial nitric oxide and reactive oxygen species, we assessed the effect of VEGF-R

Table 1
VEGF-R inhibition does not affect levels of plasma inflammatory cytokines or blood leukocyte counts.

	Placebo	PTK787	*p* value
A. Plasma cytokine profile (pg/ml)			
IFNγ	1.38 ± 0.27	1.17 ± 0.45	0.886
IL-10	49.93 ± 7.44	56.13 ± 21.87	1.000
IL-1ß	34.28 ± 3.41	28.22 ± 3.03	0.400
IL-6	25.23 ± 2.59	38.70 ± 8.91	0.200
TNFα	3.00 ± 1.15	2.34 ± 0.30	1.000
B. White blood cell count and differential (10^3/μl)			
Leukocytes	4.22 ± 0.26	5.25 ± 0.72	0.314
Lymphocytes	1.46 ± 0.22	1.50 ± 0.08	0.329
Monocytes	2.11 ± 0.30	3.18 ± 0.67	0.152
Neutrophils	0.13 ± 0.019	0.20 ± 0.13	0.407

ApoE$^{-/-}$ mice on a high cholesterol diet (1.25% wt/wt) were exposed to the pan-VEGF receptor inhibitor PTK787 for 10 weeks (50 mg/kg/d, gavage) and their whole blood or plasma was obtained for cell count or cytokine analyses. Pro- and anti-inflammatory cytokine levels **(A)** and the number of leukocytes as well as their main subpopulations **(B)** were unaltered. $n = 5$ per group, means ± SEM.

inhibition on intracellular superoxide generation; PTK787 treatment revealed a dose-dependent increase (Fig. 5D, Fig. 5G-H).

3.5. Inhibition of VEGF-R increases mitochondrial superoxide generation and decreases endothelial proliferation

Considering the magnitude of the effect of VEGF-R inhibition on intracellular superoxide generation in human aortic endothelial cells, other sources of intracellular superoxide production were assessed. No difference in NADPH oxidase activity occurred upon PTK787 treatment (Fig. 6D). However, we observed a significant dose-dependent increase in mitochondrial superoxide levels (Fig. 6A, B). In fact, endothelial mitochondria were a major source of total intracellular superoxide generation after VEGF-R inhibition in human aortic endothelial cells (Fig. 6C).

4. Discussion

4.1. Principal findings

Our data suggest the following sequence of events that link systemic VEGF-R inhibition to accelerated progression of atherosclerosis: VEGF inhibition increases mitochondrial superoxide generation in arterial endothelial cells. Resultant uncoupling of the functional eNOS homodimer leads to a deterioration of its enzymatic function and an imbalance in endothelial superoxide and nitric oxide production. The subsequent decline in the functional integrity of the endothelial monolayer accelerates the progression of pre-existing atherosclerosis. This disruption of arterial endothelial homeostasis may be one of the mechanisms underlying the cardiovascular adverse events described in recent meta-analyses of current anti-angiogenic therapies [13–16,41].

4.2. Added value of the current study

This proof-of-principle study sheds further light on the potential vascular sequelae of systemic VEGF inhibition and improves our understanding of the putative mechanisms mediating accelerated

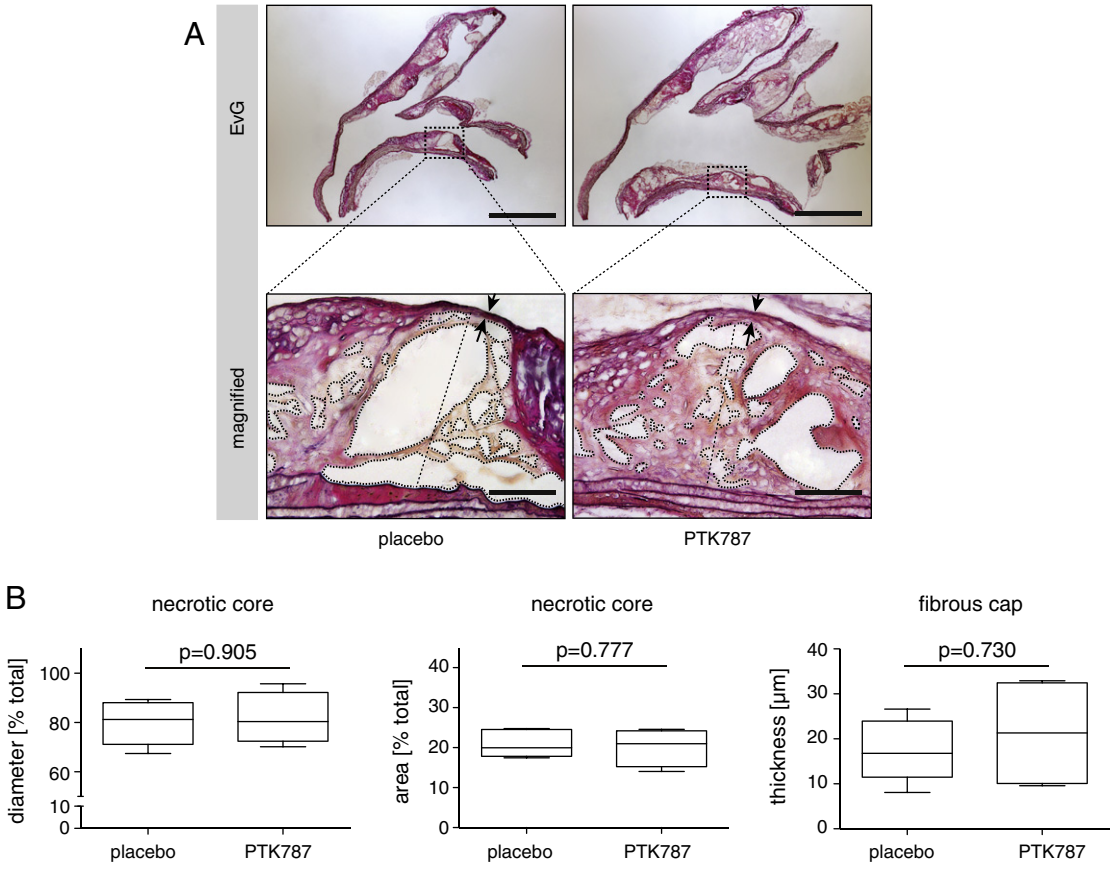

Fig. 2. *Systemic VEGF-R inhibition does not alter features of plaque vulnerability in experimental atherosclerosis*: (A) Photomicrographs of longitudinal sections of aortic arches *from ApoE*$^{-/-}$ mice treated with PTK787 or placebo; Elastic van Gieson's [EvG] stain, top: scale bars = 1 mm, dotted line indicates magnified sectors below, bottom: scale bars = 130 μm, dotted straight lines indicate necrotic core diameters, dotted curved lines indicate acellular necrotic core area, arrows indicate fibrous cap thickness. (B) Quantifications of necrotic core diameter, acellular necrotic core area, and fibrous cap thickness, $n = 5$ per group, graphs show interquartile ranges, whiskers indicate minima and maxima.

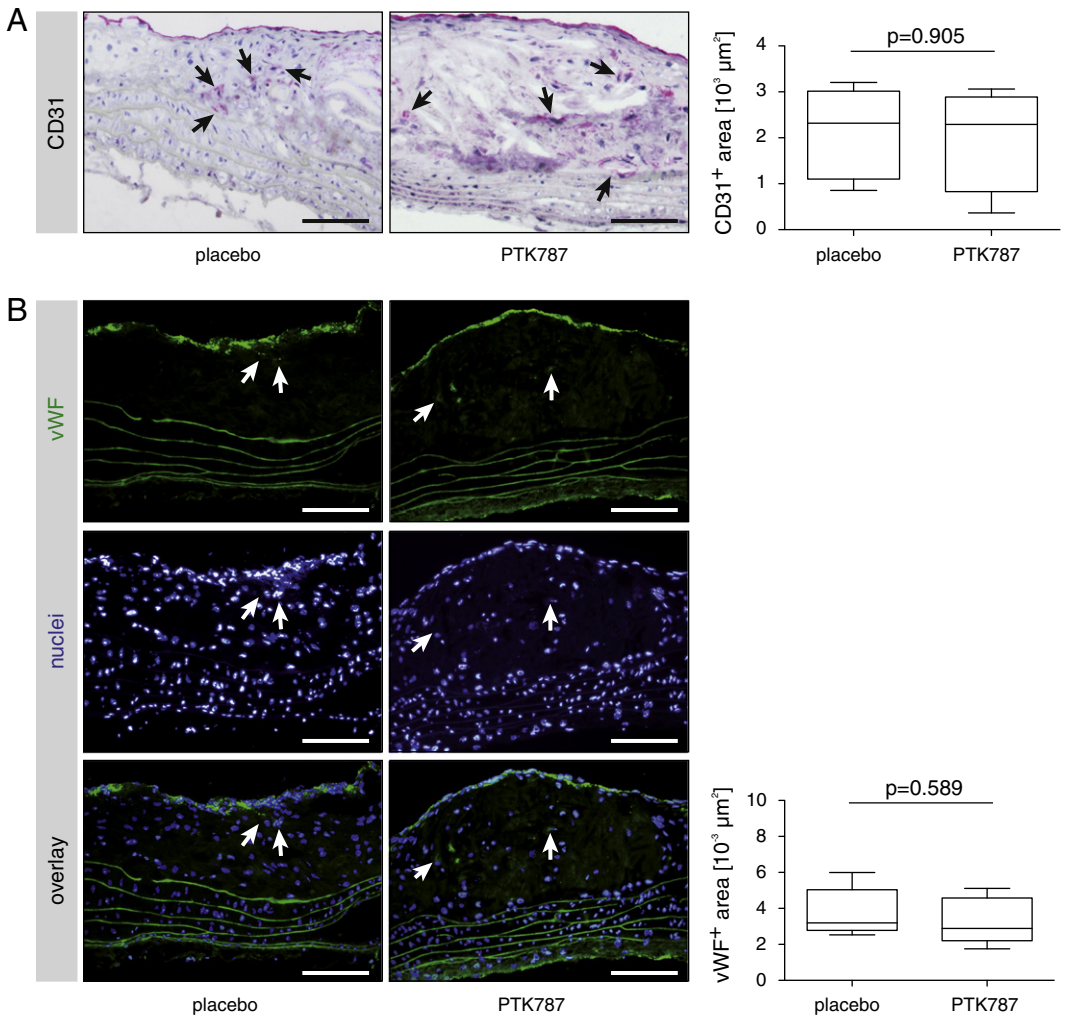

Fig. 3. *Systemic VEGF-R inhibition does not affect the number of plaque-resident endothelial cells*: Micrographs of longitudinal sections of aortic arches from *ApoE*$^{-/-}$ mice treated with PTK787 or placebo are displayed. (A) CD31 staining (purple). Arrows indicate nucleated CD31-positive signals (B) Von Willebrand Factor [vWF] staining (green), nuclei (blue). Arrows indicate nucleated vWF-positive signals. vWF-positive area was normalized to the number of nuclei. Scale bars = 100 µm, $n = 5$ per group, graphs show interquartile ranges, whiskers indicate minima and maxima.

progression of atherosclerosis in this context. Most patients undergoing anti-angiogenic treatment are aged 50 years or older as in the case of AMD, DME or RVO treatment, where average patient age is about 80 years [4,5,9,10]. Especially AMD patients are particularly prone to pre-existing atherosclerotic changes. Exposure of mice to a high-cholesterol diet before systemic VEGF-R inhibition in the current study reflects this situation of elderly patients with pre-existing atherosclerosis at the time of the initiation of VEGF-inhibiting therapy.

We have used a receptor tyrosine kinase inhibitor with a high affinity for VEGF-R2 [42] which is known to mediate pro-angiogenic signaling of VEGF-A [1]. Thus, our data represent the effects of a putative common mechanism underlying the different currently applied anti-angiogenic regimens. Qualitative analyses of atherosclerotic plaques allow the appraisal of both atherosclerotic progression (including hallmarks such as intimal macrophage infiltration and intra-plaque neoangiogenesis) and features of plaque vulnerability (i.e. fibrous cap thickness, necrotic core size). Previous findings correlate genetic or pharmacological delivery of VEGF with increased levels of NO [26,43]. Our data in which VEGF-R inhibition reduced endothelial NO release corroborate this concept. We provide additional mechanistic insight reporting an increase in mitochondrial superoxide generation and associated eNOS uncoupling in response to VEGF-R inhibition. The use of human aortic endothelial cells helps translating our findings to the human arterial endothelial lining. The dose-dependency of our results mirrors dose-dependent occurrence of clinical cardiovascular toxicities of current VEGF-R antagonists [13,44].

4.3. Important differences to other studies

We did not administer recombinant VEGF or genetically overexpress VEGF to presumably supra-physiological concentrations as has been done in previous studies [24–27,43]. In the current study, VEGF-signaling was inhibited without altering physiological VEGF concentrations, as is the case in patients receiving current anti-angiogenic regimens. Previous experimental studies have shown a VEGF-R2-mediated increase in NO levels after VEGF gene transfer using *venous* endothelial cells [26,43]. The current study substantiates these findings in a different setting, assessing the effects of VEGF inhibition in atherosclerosis-prone *arterial* vessels *in vivo* and extends mechanistic insight in human *aortic* endothelial cells. Our findings may therefore translate into the mechanisms associated with accelerated atherosclerosis and subsequent atherothrombotic events, the most threatening adverse events of current anti-angiogenic regimens [13–16,18,19].

Well-known clinical studies investigating human coronary autopsy samples have postulated that neoangiogenesis within atherosclerotic lesions, associated intra-plaque hemorrhage and macrophage

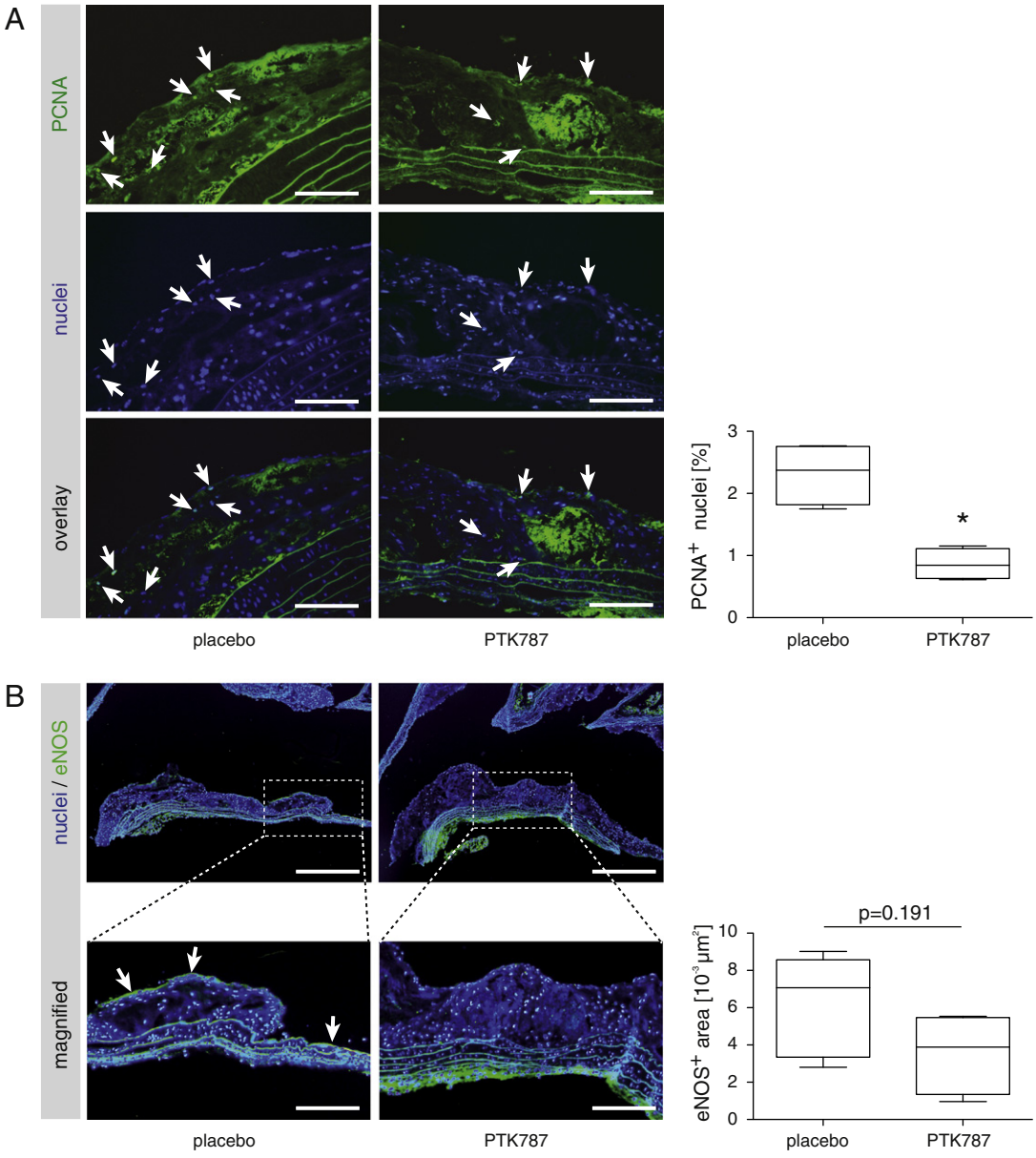

Fig. 4. *Systemic VEGF-R inhibition attenuates proliferation and endothelial eNOS expression (trend) in aortic atherosclerotic lesions*: Immunofluorescent stainings of aortic arches (longitudinal sections) from *ApoE*$^{-/-}$ mice treated with PTK787 or placebo are displayed. (A) Proliferating Cell Nuclear Antigen [PCNA] staining (green), scale bars = 50 μm (B) endothelial nitric oxide synthase [eNOS] staining (green), nuclei (blue), scale bars = 1 mm (top) and 350 μm (bottom). *$p < 0.05$, graphs show interquartile ranges, whiskers indicate minima and maxima; $n = 5$ per group.

infiltration, may accelerate the progression of atherosclerosis and the formation of unstable atheromata [29]. Accordingly inhibition of VEGF-dependent neovascularization has been reported to decrease experimental atherosclerosis [30]. Interestingly, the data of the current study cannot confirm these findings: VEGF-R inhibition accelerated athero-progression *without* affecting features of plaque vulnerability. Differences in drug effects or the experimental set-up (e.g. affecting the maturity of atherosclerotic lesions exceeding oxygen diffusion distances) may account for these opposing experimental findings. Whereas others report on increased atherosclerosis upon intraperitoneal VEGF application in different animal models [28], in several other studies no effect on atherosclerosis upon local or systemic VEGF gene transfer was observed [24,25,27]. Importantly, also no evidence of increased atherogenesis in clinical trials using recombinant VEGF or gene transfer exists [31–33]. On the contrary, a genetic polymorphism leading to increased VEGF expression was associated with atheroprotective effects [34], a finding in line with the data of the present study, and with accumulating evidence for cardiovascular adverse effects of current VEGF-inhibiting therapies. The vascular effects of VEGF appear to markedly depend on local VEGF concentrations, with low amounts necessary for vascular homeostasis and high concentrations resulting in vasculoproliferative effects [23,45]. In the context of atherosclerosis this dose-dependency may account for the opposing effects described in different settings. Importantly, outcome data of current clinical anti-angiogenic therapies indicate that inhibition of physiological concentrations of VEGF is associated with accelerated progression of atherosclerosis [13–16,18,19].

4.4. Potential limitations

This study has to be interpreted in light of the following limitations: In the mouse model of atherosclerosis used in this study, assessment of

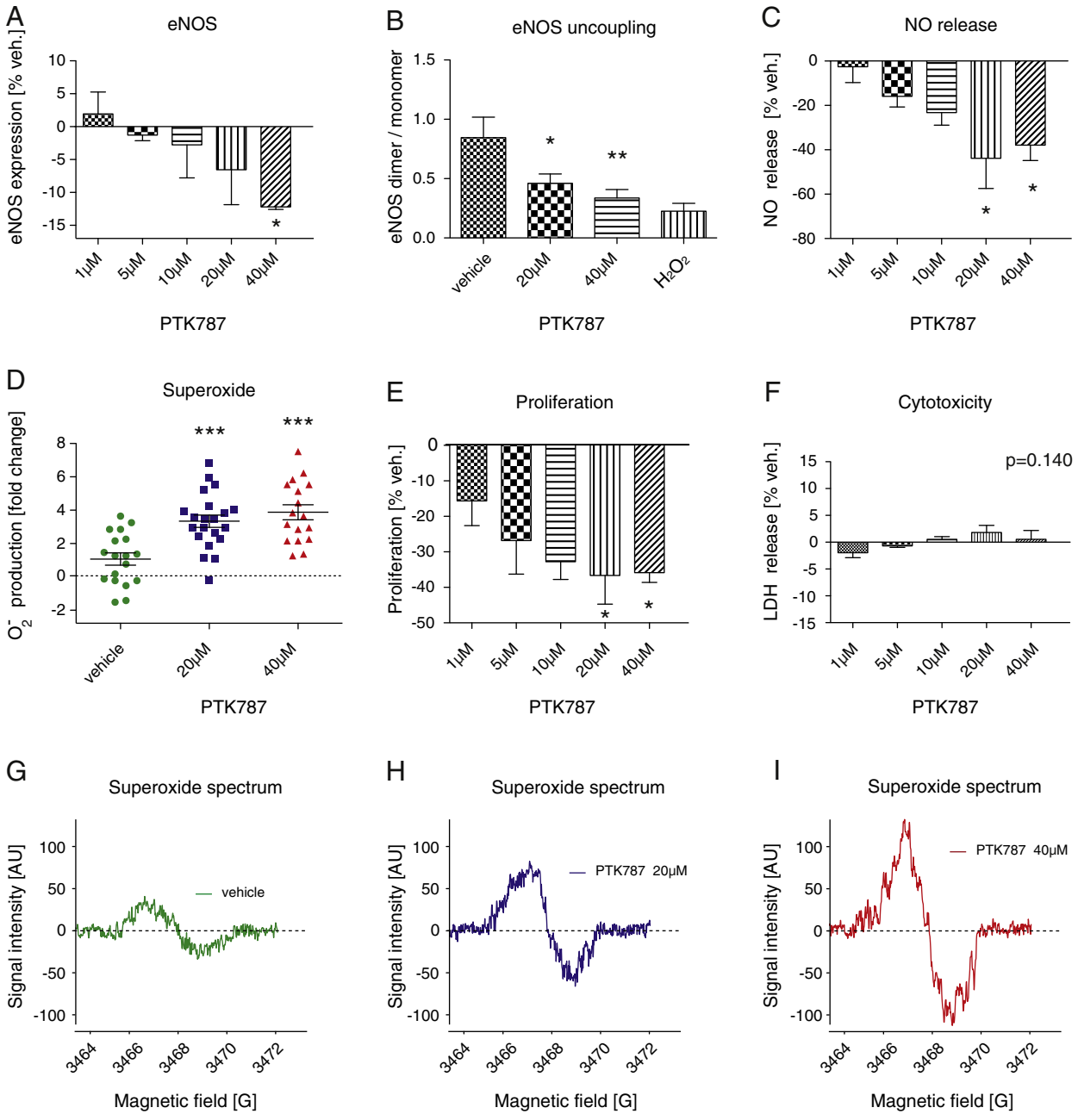

Fig. 5. *VEGF-R inhibition impairs eNOS function in human aortic endothelial cells*: Human aortic endothelial cells [HAEC] were incubated with the indicated doses of PTK787 or vehicle for 24 h and subjected to biochemical analyses. (A) Expression of endothelial nitric oxide synthase (eNOS), in-cell western analyses. (B) Expression of monomeric and dimeric eNOS, non-denaturing in-gel western analyses, hydrogen peroxide [H_2O_2] served as positive control. (C) Nitric oxide [NO] release, diaminofluorescein (DAF-2) conversion. (D) Intracellular superoxide generation, electron spin resonance [ESR] spectroscopy. (E) Endothelial proliferation, bromodeoxyuridine [BrdU] incorporation. (F) Cytotoxicity, lactate dehydrogenase release into the supernatant. (G–I) Representative ESR spectra of intracellular superoxide generation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

hypoxia-driven neoangiogenesis in the plaque center is limited since the maximal oxygen diffusion distance of about 150 μm may have not been reached. To minimize this model-based shortcoming, we exposed the mice to a prolonged high-cholesterol diet aiming for lesion diameters ≥ 150 μm. We chose this model since a range of atherosclerotic lesions very similar to the ones in human atherogenesis develops: from initial stages of simple macrophage foam cell morphology to more complex lesions consisting of acellular lipid cores and fibrous caps are seen [46]. Yet we acknowledge that advanced human atherosclerotic plaques differ from murine ones in many aspects – particularly with regard to their thickness.

The use of an agent different from the ones used in current clinical applications may limit extrapolations to the clinical side effects of one specific anti-angiogenic regimen. Based on current meta-analyses describing very similar cardiovascular adverse effects in different VEGF-inhibiting regimes, we propose a common underlying mechanism. Therefore, we chose a receptor tyrosine kinase inhibitor with a high affinity for VEGF-R2 that appears to be a common denominator of all current anti-angiogenic therapies.

4.5. Implications

In conclusion, the current study strengthens the rising concern for potentially life-threatening systemic cardiovascular side effects of current anti-angiogenic therapies both in oncology and ophthalmology. A disruption of endothelial homeostasis with subsequently accelerated

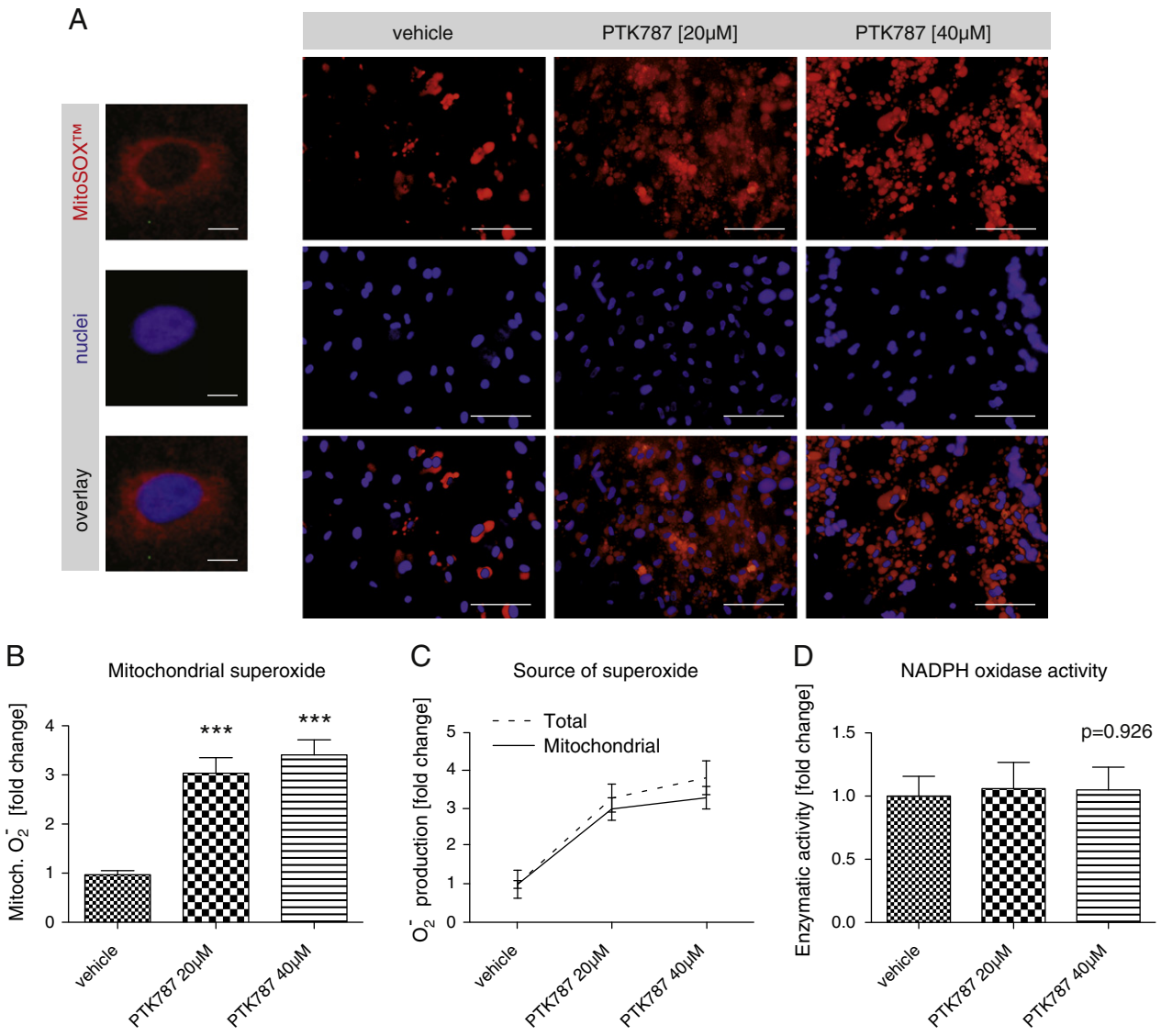

Fig. 6. *VEGF-R inhibition increases mitochondrial superoxide generation in human aortic endothelial cells*: Human aortic endothelial cells [HAEC] were incubated with the indicated doses of PTK787 or vehicle for 24 h and subjected to biochemical analyses. (A) Mitochondrial superoxide (red) generation, representative micrographs of HAEC after incubation with a fluorogenic mitochondrial-specific superoxide indicator (MitoSOX™, 5 μM, 10 min), nuclei (blue), left panel: representative magnification of a single cell. (B) Quantification of mitochondrial superoxide generation. (C) Relative comparison of total intracellular superoxide (dotted line) and mitochondrial superoxide generation (solid line). (D) NADPH oxidase activity, relative turnover of extracted NADPH. Scale bars = 50 μm, $^{***}p < 0.001$.

atherogenesis may be one of the mechanisms underlying these cardiovascular adverse effects.

Given the increased cardiovascular risk of elderly patients – representing the typical target population receiving anti-angiogenic therapies – and the increasing number of patients treated, the cardiovascular safety profile of all current anti-angiogenic regimens should be precisely determined. The identification of biomarkers for cardiovascular side effects, as exemplified by Scappaticci and colleagues in their meta-analyses including a total of 1745 patients with metastatic colorectal-, breast or non-small cell lung cancer [16], may ease patient selection for therapy and close monitoring of those at increased cardiovascular risk. Further subgroup analyses of existing randomized controlled trials and reporting of careful cardiac assessment of patients at baseline in future trials will guide the respective cardiovascular prevention in future. The development of alternative drug delivery strategies such as local gene delivery, specifically in the context of neovascular ocular diseases may reduce systemic adverse effects [47]. The cardiovascular adverse effects of currently applied VEGF-inhibiting approaches highlight the need for clinicians of different subspecialties to team up for an improved clinical outcome in an increasingly complex medical environment.

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