MS# HYPE201710490R

VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR INHIBITION INDUCES CARDIOVASCULAR DAMAGE VIA REDOX-SENSITIVE PROCESSES

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Short title: VEGF inhibition and cardiovascular toxicity

Number of figures: 6. **Abstract word count**: 250. **Total word count**: 5215

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ABSTRACT

Although vascular endothelial growth factor (VEGF) inhibitors (VEGFIs), are effective anti-cancer therapies, they cause hypertension through unknown mechanisms. We questioned whether changes in vascular redox state may be important, since VEGF signaling involves nitric oxide (NO) and reactive oxygen species (ROS). Molecular mechanisms, including NOS, Nox-derived ROS, anti-oxidant systems and vasoconstrictor signaling pathways, were probed in human endothelial cells (EC) and vascular smooth muscle (hVSMC) exposed to vatalanib, a VEGFI. Vascular functional effects of VEGFI were assessed *ex vivo* in mouse arteries. Cardiovascular and renal *in vivo* effects were studied in vatalanib- or gefitinib (epidermal growth factor inhibitor (EGFI))-treated mice. In ECs, vatalanib decreased eNOS (Ser^{1177}) phosphorylation and reduced NO and H_2O_2 production, responses associated with increased Nox-derived O_2^- and $ONOO^-$ formation. Inhibition of Nox1/4(GKT137831) or Nox1 (NoxA1ds), prevented vatalanib-induced effects. Nrf2 nuclear translocation and expression of Nrf-2-regulated anti-oxidant enzymes were variably downregulated by vatalanib. In hVSMCs, VEGFI increased Nox activity and stimulated Ca^{2+} influx and MLC_{20} phosphorylation. Acetylcholine-induced vasodilatation was impaired and U46619-induced vasoconstriction was enhanced by vatalanib, effects normalized by N-acetyl-cysteine and worsened by L-NAME. In vatalanib-, but not gefitinib-treated mice vasorelaxation was reduced and media:lumen ratio of mesenteric arteries was increased with associated increased cardiovascular and renal oxidative stress, decreased Nrf-2 activity and downregulation of anti-oxidant genes. We demonstrate that inhibition of VEGF signaling induces vascular dysfunction through redox-sensitive processes. Our findings identify Noxs and antioxidant enzymes as novel targets underling VEGFI-induced

vascular dysfunction. These molecular processes may contribute to vascular toxicity and hypertension in VEGFI-treated patients.

Keywords: Vascular endothelial growth factor, cancer, oxidative stress, vascular function, endothelial cells.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is critical in solid tumour growth and metastasis. This process is regulated by growth factors of which vascular endothelial growth factor (VEGF) plays a key role through effects on endothelial cell (EC) and vascular smooth muscle cell (VSMC) function [1]. The VEGF gene undergoes alternative splicing to form 6 isoforms, of which VEGF-A is the most biologically active [2, 3]. VEGF-A binds to two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGF receptor-2 (VEGFR-2 or Flk-1) and a non-tyrosine kinase, neuropilin (NRP1 and NRP2). However VEGFR-2 is the primary receptor through which VEGF signals to regulate angiogenesis and endothelial function [2, 4]. Binding to VEGFR-2 initiates a tyrosine kinase signaling cascade that promotes vasodilatation via nitric oxide (NO) and prostacyclins, cell proliferation/survival, migration and differentiation into mature blood vessels [5, 6].

Inhibition of angiogenesis, by targeting VEGF signaling, has revolutionized cancer therapy with improved outcomes in some previously untreatable cancers. However clinical observations unexpectedly showed that VEGF inhibition (VEGFI) was associated with cardiovascular toxicity, especially hypertension [7, 8]. The magnitude of VEGFI-induced hypertension is significant, with almost every clinical trial of VEGF inhibitors (VEGFIs)

reporting an increase in blood pressure (BP) as an adverse effect with 40-60% of patients developing hypertension often severe (>150/100 mmHg) or hypertensive crisis [9-12]. Hypertension develops acutely, within 24 hours of starting treatment, and by 6 days it is sustained [13]. Upon treatment cessation BP decreases [9, 10, 14].

The pathophysiology of VEGFI-induced hypertension is elusive, although endothelial dysfunction, vascular remodeling and capillary rarefaction have been implicated [2, 15]. VEGF is a known vasodilator through its effects on NO and as such a potential consequence of VEGFI is reduced NO, impaired vasodilatation and increased vascular tone, important determinants of augmented vascular resistance and BP elevation [16-18]. However clinical and experimental data are conflicting, with studies showing both increased and decreased eNOS activity and NO production [16, 17]. Additionally, recent findings demonstrated endothelial-independent processes are involved in VEGFI effects [19]. Increased levels of ET-1, activation of the renin-angiotensin system (RAS), EC apoptosis and rarefaction have also been implicated in VEGFI-induced hypertension [17, 18].

Oxidative stress may contribute to the development of hypertension during anti-angiogenic therapy. Recent evidence indicates that superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) play a role in VEGF/VEGFR signaling and angiogenesis [20, 21]. NADPH oxidase (Nox) isoforms are primarily responsible for vascular ROS generation in rodent (Nox1,2,4) and human vascular cells (Nox1,4,5) with Nox4 generating mainly H_2O_2 and Nox1, 2 and 5 generating O_2- [22, 23]. VEGF also regulates expression and activity of antioxidant system, including superoxide dismutase (SOD) and nuclear factor erythroid 2–related factor 2 (Nrf-2), the master regulator of antioxidant enzyme transcription [24, 25]. Thus, in addition to VEGF inducing vasodilatation through NO, generation of H_2O_2 through Nox4 and activation of

antioxidants, may influence vasorelaxation. However, whether VEGFI impacts these redox-sensitive processes to modulate a hypertensive vascular phenotype with associated BP elevation is unclear. Here we hypothesized that VEGFIs promote oxidative stress leading to impaired vasodilatation and hypercontractility, processes associated with BP elevation. Mechanisms underlying this may relate to Nox dysregulation, downregulation of Nrf-2 -regulated antioxidant systems and altered Ca^{2+} handling in vascular cells.

METHODS

The authors declare that all supporting data are available within the online supplementary files **(Please see http://hyper.ahajournals.org. for expanded Methods section).**

All experimental protocols on mice were performed in accordance with the Ethical Principles in Animal Experimentation adopted by the West of Scotland Research Ethics Service and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Studies at Sapienza University were conducted in accordance with the Italian Law on the Protection of Animals. Human vascular smooth muscle cells were isolated from surgical specimens and in accordance with protocols approved by the West of Scotland Research Ethics Service (WS/12/0294).

Experimental models

Studies were performed at the cellular (human ECs, VSMCs), tissue (isolated mouse arteries) and whole animal (VEGFI-treated mice) levels. We examined effects of a VEGFR inhibitor, vatalanib and in some experiments compared effects to gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR). We used this comparator agent because VEGF and EGF signal

through similar pathways, yet whereas VEGF inhibition causes hypertension, EGFR inhibition does not.

Cell culture. Cell-based studies were performed in human aortic endothelial cell (HAEC) and primary culture vascular smooth muscle cells (hVSMC).

Mice. ***Ex vivo vascular studies.*** In some experiments, mouse mesenteric arteries were isolated to assess vascular functional responses to vatalanib in the absence and presence of L-NAME (eNOS inhibitor) or N-acetyl-cysteine ((NAC) ROS scavenger). ***In vivo studies:*** Three groups of male SV-129 mice were studied for 2 weeks): 1) vehicle-treated group; 2) VEGFR inhibitor, vatalanib-treated group (Vat, 100 mg/Kg/day), and 3) EGFR inhibitor, gefitinib-treated group (Gef, 100 mg/Kg/day).

Experimental protocols

Vascular levels of NO, O_2^-, H_2O_2 and nitrotyrosine and Nox activity were assessed by fluorescence, chemiluminescence and amplex red assays. VSMC $[Ca^{2+}]_i$ was measured by Cal-520 fluorescence.

Nrf-2 activity was assessed by nuclear translocation and Keap-1 expression by immunoblotting. Anti-oxidant enzymes (catalase activity) and gene expression (SOD1, catalase, GPX1, HO1) were determined by activity assays and qPCR.

Phosphorylation of eNOS and MLC20 was determined by immunoblotting.

Vascular functional and structural properties were assessed by wire and pressure myography.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as means±standard error of the mean (SEM). Groups were compared using student's *t* test or one-way analysis of variance (ANOVA). Bonferroni or Tukey's post-test were used as appropriate. Results of statistical tests with $p<0.05$ were considered significant.

RESULTS

Vatalanib influences ROS and NO generation in human endothelial cells

Vatalanib increased NADPH-dependent O_2^- generation in HAECs, effects that were inhibited by GKT137831 and NoxA1dstat (**figure 1A**). This was associated with increased p47phox membrane expression (**figure 1B**), reduced generation of H_2O_2 and NO and increased formation of $ONOO^-$ (**figures 1C, 1D, S1**). In vatalanib-treated cells, phosphorylation of eNOS (active site, Ser^{1177}) was reduced (**figure S1**). Vatalanib, at 24 hours, influenced expression of endothelial cell Noxs, which are responsible for O_2^- production. In particular vatalanib decreased expression of Nox4 and increased expression of Nox5 (**figure 2A, 2B**), without significantly influencing Nox1 (**figure S2**). Nuclear accumulation of Nrf2 and gene expression of Nrf-2-regulated anti-oxidant genes, catalase, GPX1 and HO1, but not SOD1, was downregulated 8 hours after vatalanib treatment **(figure 1E, 2C-F).** Gefitinib does not alter O_2^- production neither modulates Noxs and anti-oxidants mRNA levels in vascular cells (**figure S6**).

Vatalanib increases ROS generation and modulates pro-contractile signaling in human vascular smooth muscle cells

Vatalanib increased O_2^- production and $ONOO^-$ levels in hVSMC (**figure 1F, S1**). Pretreatment with Nox1/4 inhibitors prevented vatalanib-induced ROS generation. VEGF inhibition induced a

significant increase in Ca^{2+} influx in hVSMCs, effects that were attenuated by N-acetyl-l-cysteine (NAC) (**figure 3E**). Vatalanib also influenced pro-contractile signaling pathways, by inducing phosphorylation of MLC_{20}, critically involved in triggering vascular contraction (**figure 3D**).

Vascular dysfunction induced by vatalanib is mediated by NOS- and redox-sensitive mechanisms

To evaluate whether vatalanib-induced effects observed at the cellular level have functional significance at the vascular level, we studied isolated mouse mesenteric resistance arteries by myography and exposed vessel segments to vatalanib in the absence and presence of NOS inhibitors and ROS scavengers. As shown in figure 3A, ACh induced almost 100% vasorelaxation in control vessels, whereas in vatalanib-treated vessels, ACh-mediated vasorelaxation was reduced, with arteries relaxing maximally ≈ 40%. These responses were worsened by L-NAME, a NOS inhibitor. In arteries pre-treated with NAC, vatalanib-induced endothelial dysfunction was ameliorated (**figure 3B**). Corroborating our findings in hVSMC, vatalanib amplified agonist (U46619)-induced vasoconstriction, an effect blocked by NAC (**figure 3C**).

Systemic oxidative stress, vascular dysfunction and arterial remodeling in vatalanib-treated mice.

To further explore whether vatalanib influences redox-sensitive processes and vascular function *in vivo*, we examined mice treated with vatalanib for 2 weeks, and compared effects to gefitinib, an EGFR inhibitor. At the doses used, mean blood pressure was not significantly different in control (92.6±1.7 mmHg), vatalanib-treated (91.2±1.8 mmHg) and gefitinib-treated groups

(88.0±2.5 mmhg). Vatalanib increased systemic ROS generation, as indicated by elevated plasma TBARS levels in the vatalanib (9.0±2.0 µmol/l) versus vehicle (5.1±0.2 µmol/l) and gefitinib groups (5.2±0.5 µmol/l).

As shown in **figure 4A** and supplemental table S1, ACh-induced maximal vasorelaxation and EC_{50} of isolated small mesenteric arteries were blunted in vatalanib- but not gefitinib-treated mice. SNP-induced vasodilatation was not influenced by either agent (**figure 4B**). Mesenteric arteries from vatalanib-treated mice also exhibited an increase in media-to-lumen ratio indicating vascular remodeling **(figure S3).** Vatalanib had no effect on cross-sectional area (CSA) **(figure S3).** Gefitinib did not significantly influence vascular function or structure.

Vatalanib modulates redox signaling in tissues from mice

To determine whether VEGFIs influence redox status in cardiovascular and renal tissue, we assessed levels of H_2O_2, O_2- and $ONOO^-$, catalase activity and expression of pro-oxidant oxidases and anti-oxidant enzymes in aorta, kidney and heart in VEGFI- treated mice.

As shown in figure 4 aortic and cardiac levels of H_2O_2 were reduced, while $ONOO^-$ levels were increased. Catalase activity was increased in aorta in the vatalanib group. Gene expression of Nox1, but not Nox2 or Nox4, was significantly increased in the heart by vatalanib and gefitinib (**figure S4**). Cardiac gene expression of anti-oxidant enzymes catalase and GPX1 was reduced in vatalanib-treated mice, without effect on SOD1 (**figure S4**).

NADPH-stimulated production of O_2^- and H_2O_2 levels were augmented by vatalanib in kidneys (**figures 5A, 5B**). This was associated with decreased activity of renal catalase **(figure 5C)** and downregulation of the master anti-oxidant transcription factor Nrf2, indicated by decreased Nrf2 nuclear translocation and increased cytosolic levels of the Nrf2 repressor, Keap-1 (**figures 5D, 5E**). At the gene level, expression of anti-oxidant enzymes catalase and GPX1

(**figures 5F)**, but not SOD1, was reduced in treated mice **(figure S5).** Vatalanib decreased mRNA expression of Nox4, without effect on Nox1 and Nox2 **(figure S5).**

DISCUSSION

Despite the anti-angiogenic and anti-cancer benefits of VEGF inhibitors in clinical medicine, these agents have potent vascular toxicities and are pro-hypertensive due to, as yet, unclear molecular mechanisms. Processes that have been implicated include reduced endothelial-derived NO production, increased ET-1 levels, activation of the renin-angiotensin system, endothelial cell apoptosis and microvascular rarefaction [26 16 17]. Here we advance the field by demonstrating an important role for oxidative stress. In particular, we show that vatalanib, a VEGFR inhibitor, increased vascular cell ROS production and $ONOO^-$ formation and decreased activation of the eNOS-NO pathway. These phenomena translated to endothelial dysfunction, vascular hypercontractility and cardiovascular and renal oxidative stress in VEGFI-treated mice. Potential mechanisms underlying these effects involve upregulation of Noxs, in an isoform- and tissue-specific manner, and downregulation of Nrf2-regulated anti-oxidant genes.

Endothelial function and vascular integrity are regulated by VEGF/VEGFR, through multiple signaling pathways, including PI3K-NOS and protein tyrosine phosphatases (PTP), which are modulated by changes in redox state. In particular VEGF-induced activation of NOS and PTPs is linked to a reduced oxidative milieu that maintains vascular health [20, 21, 27, 28]. In addition, signaling through VEGF protects cells from oxidative stress, in part through activation of Nrf2-regulated antioxidant enzymes [29, 30]. Disruption of these protective systems by inhibiting VEGF signaling leads to oxidative stress and cell damage. We explored this concept in the context of VEGFI-induced vascular toxicity and investigated whether vatalanib, a VEGFR inhibitor,

influences redox state in human endothelial and vascular smooth muscle cells. Since we were particularly interested in the direct cellular effects of VEGFI, recapitulating the clinical scenario of anti-angiogenic therapy, our studies were conducted without adding exogenous VEGF. Both endothelial cells and VSMCs exhibited increased vatalanib-induced NADPH-stimulated O_2^- production, involving Nox1/Nox4. Since ROS generation was rapid, it is likely that constitutively functional Nox1/4 was modulated by vatalanib, which also had more long-term actions by regulating Nox gene expression in an isoform-specific manner. The acute effect may also relate to dampening of protective anti-oxidant systems by vatalanib, similar to what has been shown for other VEGFIs [31, 32]. In ECs, a consequence of increased O_2^- production is decreased eNOS-generated NO bioavailability and increased $ONOO^-$ formation, as we observed. These events, together with reduced generation of H_2O_2, which induces vasodilation and is vasoprotective [33, 34], may underlie endothelial dysfunction and vascular oxidative damage by vatalanib. In support of our findings, others have shown that VEGFIs acutely increase ROS generation in retinal pigment epithelial cells [29], increase oxidative cellular toxicity [35] and augment oxidative stress, inflammation and endothelial dysfunction in mouse lung and human lung microvascular endothelial cells [36].

To investigate whether the findings observed at the cellular level translate to functional responses, we studied mouse vessels exposed to vatalanib *ex vivo*, and demonstrated significantly impaired endothelial function, responses that likely involve dysregulated eNOS and oxidative stress because L-NAME worsened vasorelaxation, whereas the ROS scavenger NAC, ameliorated endothelium-dependent vasorelaxation.Vatalanib also amplified agonist-stimulated vasoconstriction, possibly linked to increased $[Ca^{2+}]_i$ signaling and activation of contractile machinery, as evidenced by increased phosphorylation of MLC as we demonstrated in VSMCs.

Since NAC normalized hypercontractile responses, redox-sensitive processes are likely also important in vatalanib vascular functional effects. In line with our findings, four multi-targeted VEGFIs potently increased vasoconstriction in mice [19].

The findings in isolated arteries were recapitulated in *in vivo* studies in mice treated for 2 weeks with vatalanib, where endothelium-dependent vasorelaxation was attenuated with associated increased cardiovascular production of O_2^- and $ONOO^-$ and reduced generation of H_2O_2. Whereas O_2^- and $ONOO^-$ are associated with vasoconstriction and vascular injury, H_2O_2 is vasoprotective acting as a vasodilator through protein kinase G (PKG) [33, 34, 37]. Processes underlying these phenomena likely involve decreased eNOS/NO generation, Nox activation, increased catalase activity (which catalyzes the decomposition of H_2O_2 to H_2O and O_2) and reduced protective anti-oxidant systems, similar to what we observed in human vascular cells.

Whereas vatalanib reduced H_2O_2 production in vessels, it increased production in kidneys, possibly due to reduced catalase activity. Renal oxidative stress was increased by vatalanib, with associated decreased activity of Nrf2 as evidenced by decreased nuclear translocation and increased cytosolic content of the Nrf2 repressor Keap-1. Loss of Nrf2 activity, which regulates anti-oxidant genes, such as catalase and GPX1, likely dampens the antioxidant protective status, contributing to renal oxidative stress in vatalanib-treated mice. Interplay between VEGF, Nrf2 and other antioxidant systems has been demonstrated [58,59] and a VEGF-Nrf2 positive feedback loop, which protects against oxidative stress, has been demonstrated in brain microvascular endothelial cells [38] and in cancer cell lines [30]. Hence disruption of this feedback loop with VEGFIs, would downregulate Nrf2, similar to what we observed in our studies. Aggravation of renal damage by VEGFIs has also been shown in diabetic mice, processes attributed to oxidative stress and inactivation of the Akt/eNOS/NO axis [39].

To elucidate whether inhibition of VEGFR tyrosine kinases by vatalanib is a generalized or specific phenomenon, we also evaluated effects of gefitinib, which inhibits EGFR, in part, through common VEGFR signaling pathways. Our findings clearly demonstrate that vatalanib, but not gefitinib induced vascular dysfunction and cardiovascular and renal oxidative stress, suggesting specific cardiovascular toxicity when VEGFR is targeted. Others have also shown differential effects of VEGFI and EGFIs [28,29]. Mice treated with sunitinib, which primarily targets VEGFR tyrosine kinases, exhibited systolic dysfunction and metabolic abnormalities, which were absent in mice treated with the EGFRI erlotinib [40]. Using a non-targeted metabolomics approach, it was found that sunitinib, but not erlotinib, decreased docosahexaenoic acid (DHA), arachidonic acid (AA)/ eicosapentaenoic acid (EPA), O-phosphocolamine, and 6-hydroxynicotinic acid, important anti-inflammatory mediators and regulators of mitochondrial function. Loss of these compounds may underlie VEGFI-induced mitochondrial dysfunction, oxidative stress and cardiovascular damage [40].

Despite the vascular dysfunction, arterial remodelling and significant cardiovascular and renal oxidative effects induced by vatalanib, mice did not develop hypertension. Reasons for this may relate to the low dose of vatalanib used and/or to the relatively short treatment period. In addition, we used tail cuff methodology to measure blood pressure at one time point, and as such we may have missed subtle changes in blood pressure, especially over the 24-hour period. Telemetry would have provided a better approach to fully characterise blood pressure changes. Nevertheless, our data clearly demonstrate, that even at sub-pressor doses, vatalanib induced cardiovascular and renal toxicity, which may be amplified with higher doses and more chronic treatment [39].

In conclusion, this study provides novel mechanistic insights to better understand the pathophysiology of VEGFI-induced vascular dysfunction and hypertension. In particular, we demonstrate at the cellular, vascular and whole animal levels, that vatalanib promotes oxidative stress, Nox dysregulation and downregulation of Nrf2-regulated antioxidant systems. Our study identifies redox-sensitive mechanisms whereby VEGF signaling inhibition may cause cardiovascular toxicity, as highlighted in figure 6. This study might be especially important to guide new therapeutic approaches to reduce cardiovascular risk without compromising anti-cancer benefit of VEGFIs. Such an approach may include strategies to reduce VEGFI-induced oxidative stress using adjuvant therapies such as Nrf2 activators or Nox inhibitors. This concept awaits further confirmation.

PERSPECTIVES

Our results identify novel molecular mechanisms involving changes in redox state whereby VEGFI promotes vascular injury and dysfunction. Our data are of clinical significance because these processes may contribute to vascular toxicities associated with VEGFI-associated hypertension in patients treated with anti-angiogenic therapy targeting VEGF signaling pathways. In particular our findings that VEGFI-indiced oxidative stress is linked to upregulation of vascular Noxs and dampening of anti-oxidant enzymes may direct future therapeutic approaches to reduce vascular toxicities caused by VEGFI anti-cancer drugs. For example, adjuvant therapy with Nrf2 agonists may be an interesting approach, that might warrant further consideration.

SOURCES OF FUNDING

This study was funded by grants from the British Heart Foundation (BHF) (RE/13/5/30177) and Research Award, Sapienza University of Rome. RMT is supported by a BHF Chair (CH/12/429762). CS was supported by Fondazione Roma (NCDS-2013-00000345) and the Italian Ministry of Education, University and Research (PRIN-2015ZTT5KB 003).

ACKNOWLEDGEMENTS

The authors thank Emanuele Arrabito and Carmine Nicoletti for their technical support.

DISCLOSURES

None.

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NOVELTY AND SIGNIFICANCE

What Is New?

This study demonstrates that vatalanib, a VEGFR inhibitor, promotes oxidative stress by upregulating Noxs and inhibiting Nrf-2-regulated anti-oxidant systems. These processes contribute to VEGFI-induced vascular dysfunction.

What Is Relevant?

- Molecular mechanisms underlying VEGFI-induced vascular toxicity and hypertension are unclear, but oxidative stress may be important.
- Modulating redox-sensitive targets of VEGFIs may ameliorate vascular dysfunction and injury associated with anti-angiogenic therapy.
- Inhibiting oxidative stress by decreasing Nox activity or activating Nrf-2-regulated anti-oxidant systems during VEGFI treatment may prevent cardiovascular risk and hypertension, without compromising anti-cancer therapy.

Summary

We identify novel molecular mechanisms involving redox-dependent processes whereby VEGFIs cause endothelial dysfunction and vascular injury. In particular, we demonstrate at the cellular, vascular and whole animal levels, that the VEGFI vatalanib promotes oxidative stress, Nox dysregulation and downregulation of Nrf2-regulated antioxidant systems. These processes may play a role in vascular toxicity and hypertension in patients treated with VEGFI anti-angiogenic therapy.

FIGURE LEGENDS

Figure 1. Vatalanib influences ROS and NO generation and modulates pro-contractile signaling in human vascular cells. (A) Lucigenin-derived chemiluminescence was performed in HAEC stimulated with vehicle or vatalanib in the presence or absence of NoxA1ds or GKT137831. (B) p47 phox membrane expression was assessed by western blotting in HAEC in presence of vatalanib or vehicle. (C) H_2O_2 levels, measured by amplex red in HAEC exposed to vatalanib. (D) NO levels were determined by DAF-FM in vatalanib-treated HAEC (5 minutes) in the presence or absence of GKT137831 or NoxA1ds (30 minutes). (E) Nuclear accumulation of Nrf2 was determined by ELISA in HAEC nuclear extracts. (F) Lucigenin-derived chemiluminescence was performed in hVSMC stimulated with vehicle or vatalanib±NoxA1ds or GKT137831. Results from amplex red, lucigenin and DAF-FM assays were normalized by protein content. Results are means±SEM of 4 to 7 experiments. *p<0.05 *vs*. control; # *vs*. vatalanib.

Figure 2. Expression of pro-oxidant (Noxs) and anti-oxidant systems in human vascular cells is modulated by vatalanib. mRNA expression of the Nox isoforms Nox4 (A), Nox5 (B), and the anti-oxidant genes catalase (C), GPX1 (D), HO1 (E) and SOD1 (F) was determined by real time PCR in HAECs. PCR values were normalized by GAPDH mRNA expression. Results are mean±SEM of 4-6 experiments. *p<0.05 *vs*. control.

Figure 3. Vascular dysfunction induced by vatalanib is mediated through mechanisms involving oxidative stress and downregulation of eNOS. Endothelium-intact mesenteric resistance arteries from wild type mice were incubated with vatalanib (100 nmol/L) or vehicle for 30 min. Relaxation responses of Phe-contracted vessels to ACh was evaluated in the absence and presence of (A) L-NAME (eNOS inhibitor, 100 µmol/L) or (B) N-acetyl-cysteine ((NAC),

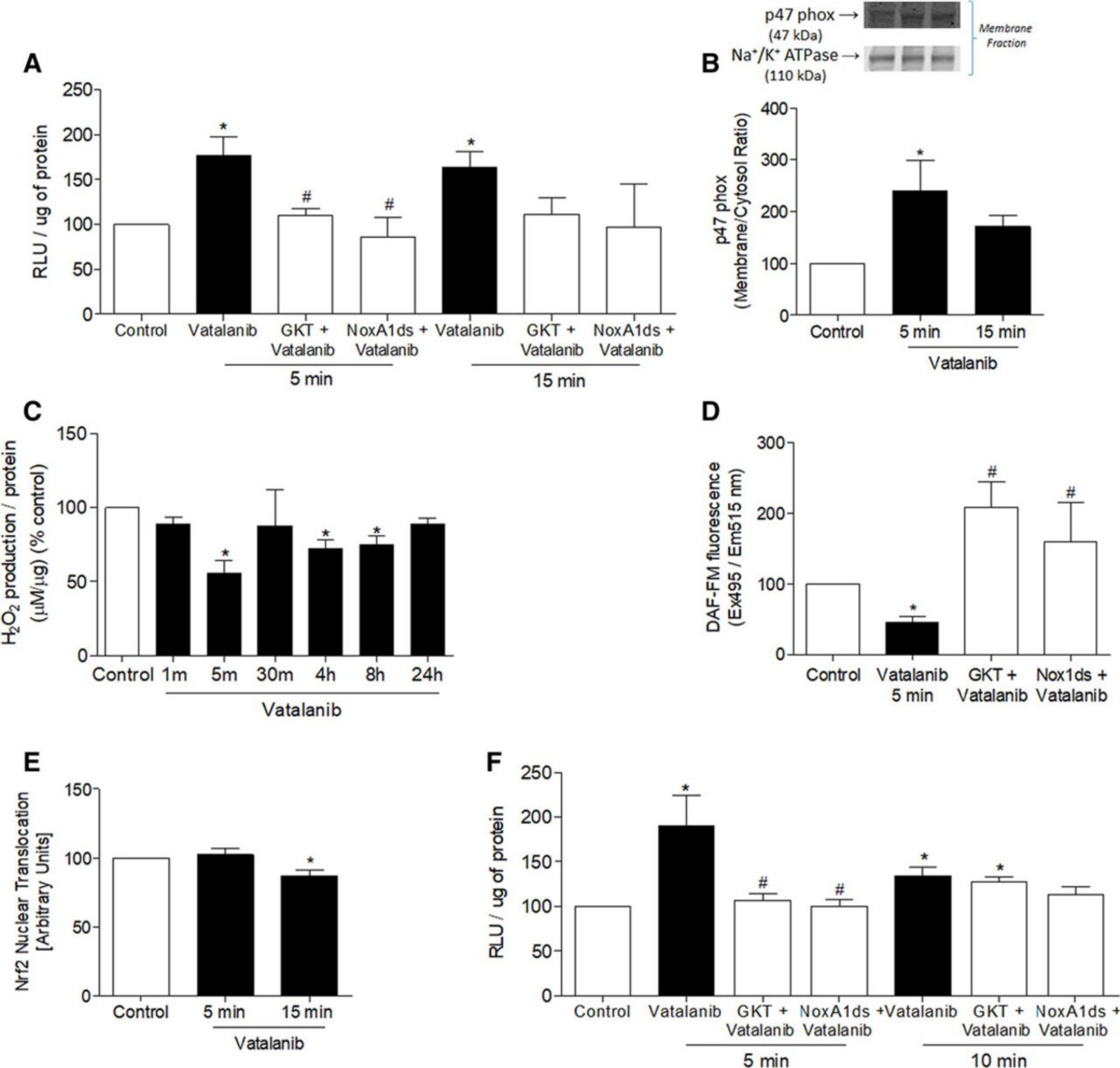
ROS scavenger, 10 μmol/L) was added 30 min before vehicle or vatalanib addition. (C) Contraction curves to U46619 in the absence or presence of NAC. (D) Phosphorylation of MLC_{20} (Thr^{18}/Ser^{19}) was determined byimmunoblotting in hVSMC stimulated with vatalanib (100 nmol/L); values were normalized by α-tubulin expression. (E) Ca^{2+} influx was assessed in hVSMC exposed to vatalanib ± NAC. Area under the curve (AUC) indicates global Ca^{2+} influx in hVSMC. Results are mean±SEM of 3-6 experiments. *p<0.05 *vs.* vehicle; # p<0.05 *vs.* vatalanib; ω p<0.05 *vs.* L-NAME.

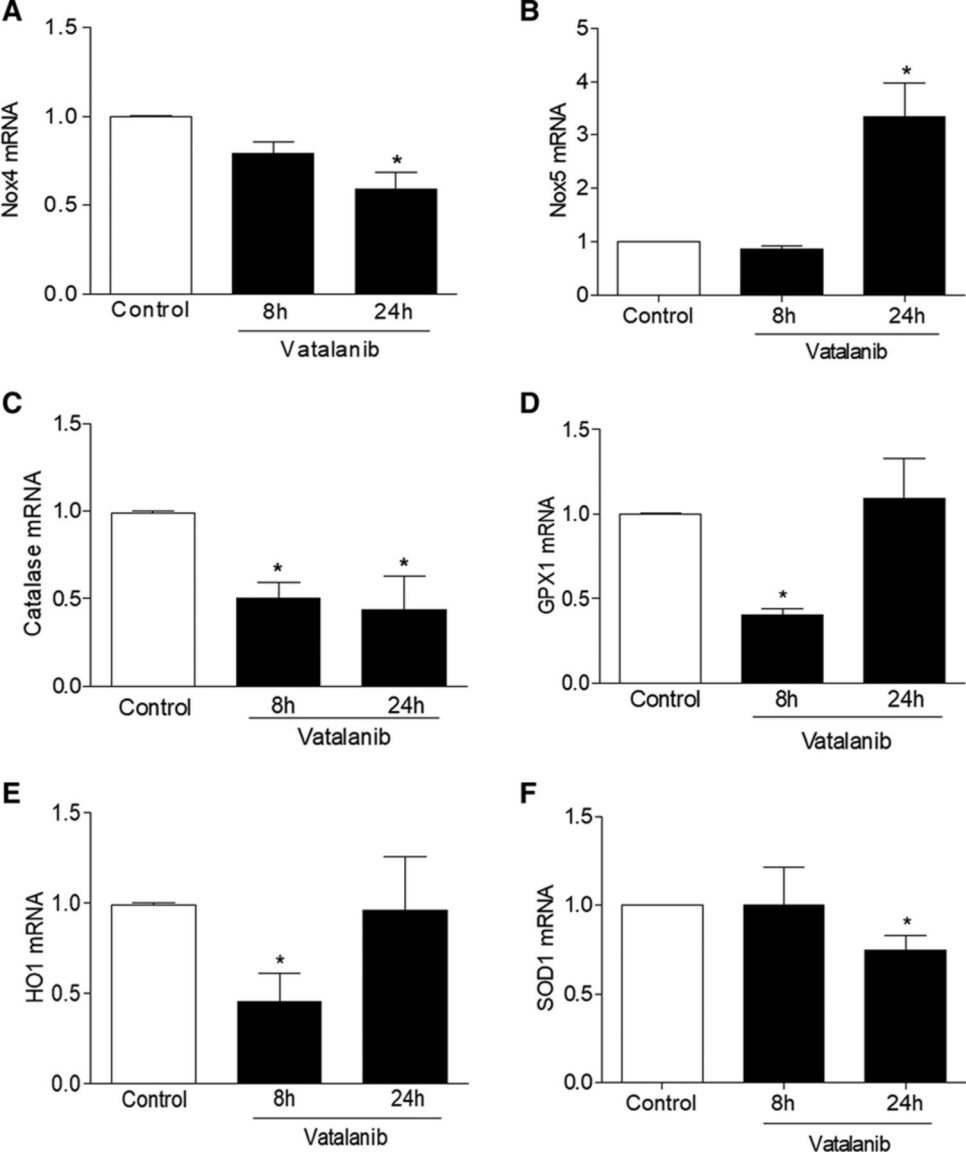
Figure 4. Vatalanib, but not gefitinib, impairs ACh-induced vasodilation and modulates redox signaling in aorta and heart from mice. Bar graphs represent the maximal response (E_{max}) of ACh (A) and SNP (B) in mesenteric arteries from mice treated with vatalanib (100 mg/Kg/day), gefitinib (100 mg/Kg/day) or vehicle assessed by wire myograph. H_2O_2 levels were measured by amplex red assay in endothelium-intact aorta and heart (C) from vatalanib and gefitinib-treated mice. Nitrotyrosine was assessed as an index of peroxynitrite ($ONOO^-$) formation in aorta and heart (D) from treated mice. Catalase activity was performed in aorta and heart E) by assay kit. Results were normalized by protein content. Results represent the mean±SEM of 5-7 experiments. *p<0.05 *vs.* vehicle.

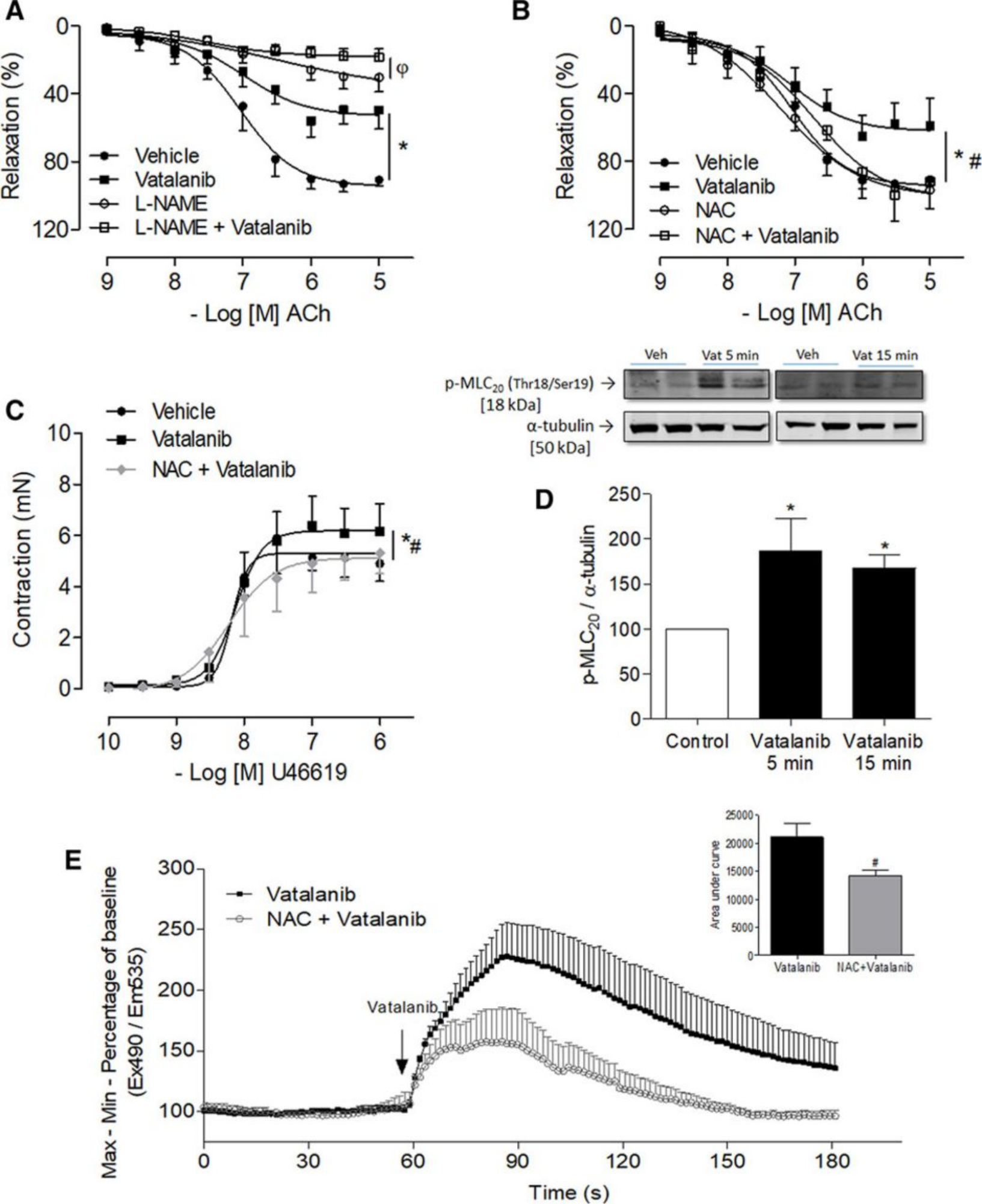
Figure 5. Renal oxidative and anti-oxidant effects of vatalanib. (A) NADPH-stimulated O_2^- production, assessed by lucigenin assay, in kidneys from vatalanib and gefitinib-treated mice. (B) H_2O_2 levels and catalase activity (C) measured by amplex red assay in kidneys. (D) Nuclear accumulation of Nrf2 was determined by ELISA in kidney nuclear extracts and (E) cytosolic Keap-1 cytosolic protein expression was evaluated by immunoblotting. (F) mRNA expression of Nrf2-regulated genes (catalase, GPX1) was determined by real time PCR. PCR values were

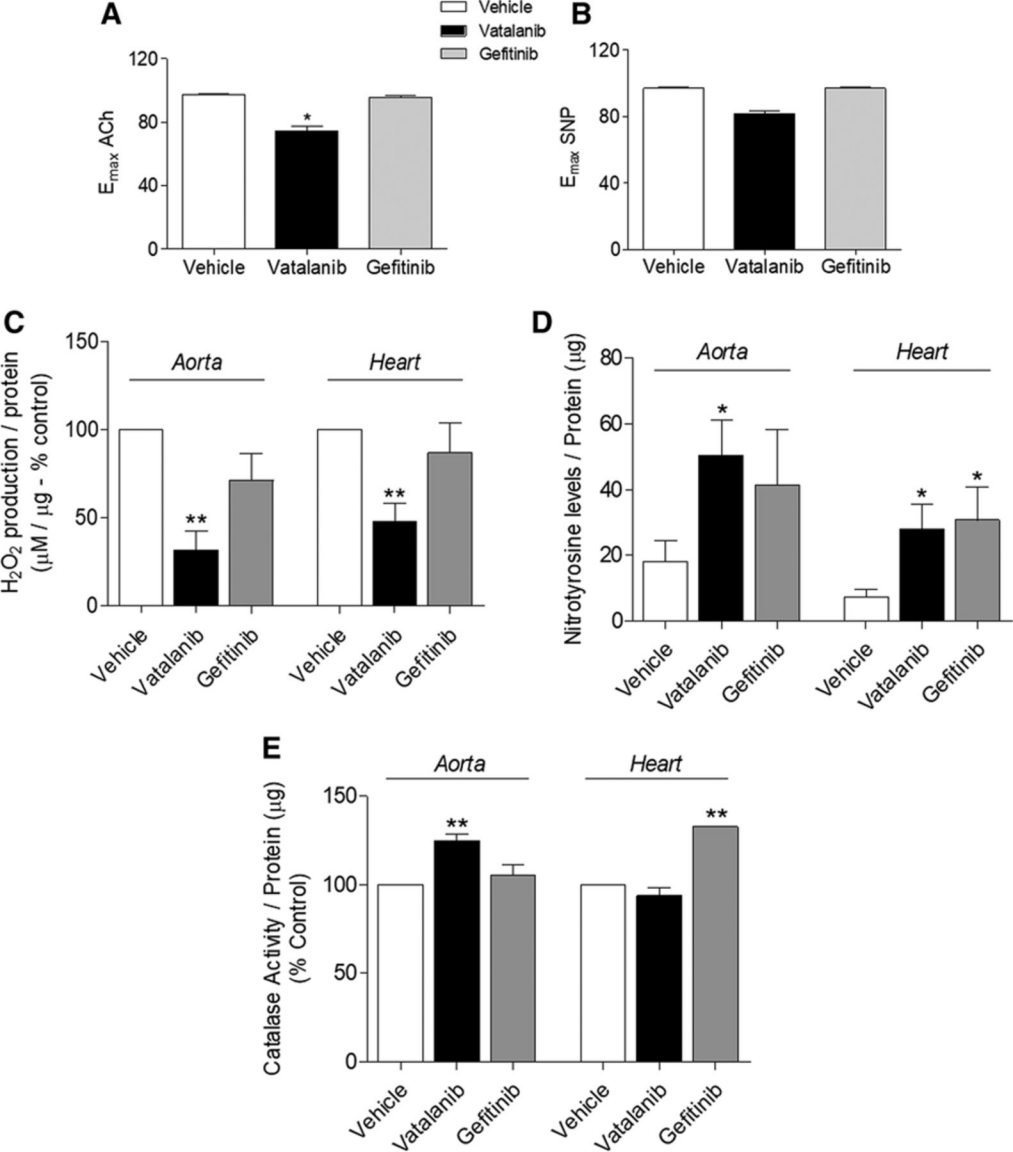
normalized by GAPDH mRNA expression and Keap-1 values were normalized by α-tubulin protein expression. Results are mean±SEM of 5-7 experiments. *p<0.05 *vs*. vehicle.

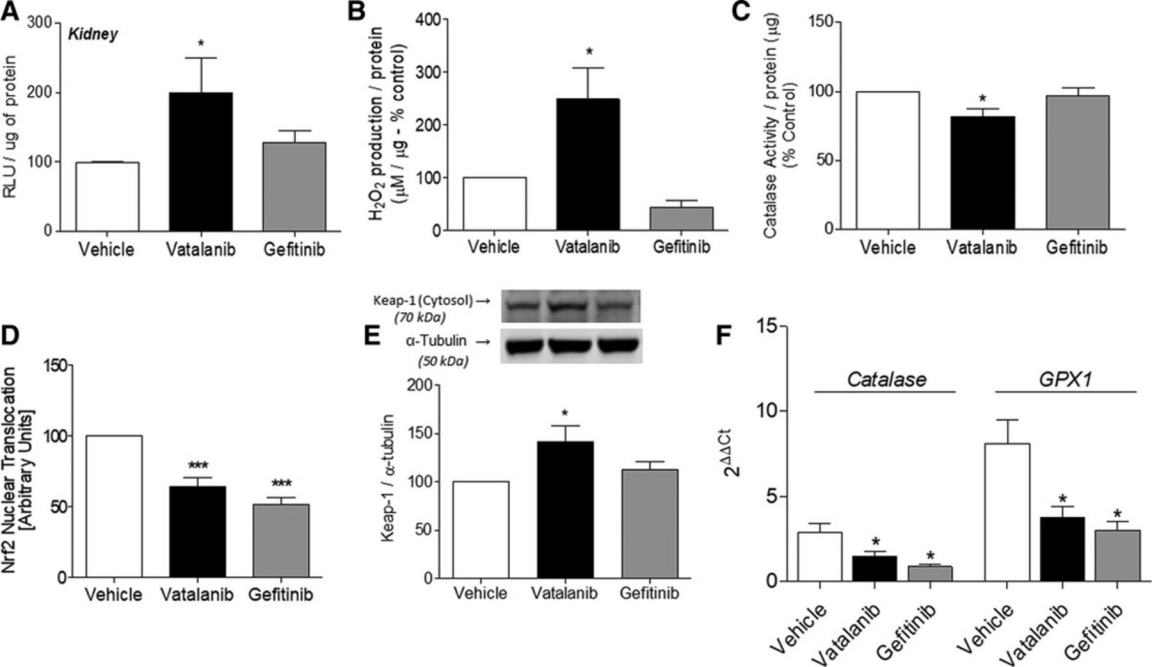
Figure 6. Putative mechanisms involving redox-sensitive processes whereby VEGF/VEGFR inhibition impacts vascular smooth muscle and endothelial cell function. VEGF inhibition by vatalanib in hVSMC (left panel) causes an increase in ROS generation through Nox1 and 4 activation which is involved in pro-contractile signaling, such as increased $[Ca^{2+}]_i$ and activation of MLC_{20}, leading to enhanced vascular contraction. In endothelial cells (right panel), vatalanib acts by increasing ROS production through Nox activation but also by downregulating the antioxidant system. In addition, vatalanib decreases the vasodilatory ROS, H_2O_2, as well as reduces eNOS phosphorylation and NO production in endothelial cells, which may be culminating in endothelial dysfunction. The dysregulation of both vascular smooth muscle and endothelial cell function induced by vatalanib may induce vascular tone alterations and vascular remodeling, and may explain, at least in part, molecular mechanisms underlying VEGFI-associated hypertension.

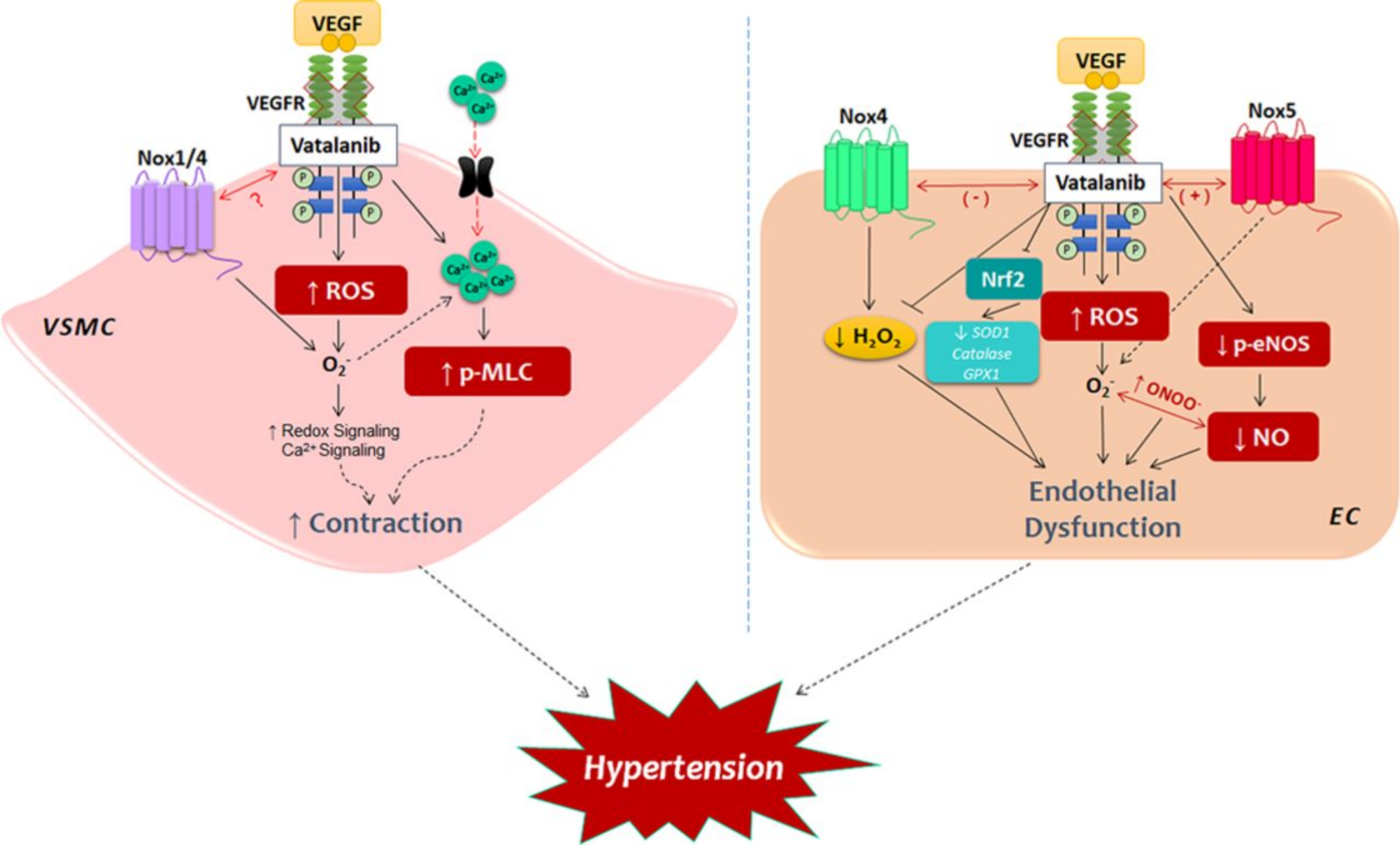

A
RLU / ug of protein
250
200
150
100
50
0
Control
Vatalanib
GKT + Vatalanib
NoxA1ds + Vatalanib
Vatalanib
GKT + Vatalanib
NoxA1ds + Vatalanib
5 min
15 min
B
p47 phox →
(47 kDa)
Na+/K+ ATPase →
(110 kDa)
Membrane Fraction
p47 phox (Membrane/Cytosol Ratio)
400
300
200
100
0
Control
5 min
15 min
Vatalanib
C
H2O2 production / protein (μM/μg) (% control)
150
100
50
0
Control
1m
5m
30m
4h
8h
24h
Vatalanib
D
DAF-FM fluorescence (Ex495 / Em515 nm)
300
200
100
0
Control
Vatalanib 5 min
GKT + Vatalanib
Nox1ds + Vatalanib
E
Nrf2 Nuclear Translocation [Arbitrary Units]
150
100
50
0
Control
5 min
15 min
Vatalanib
F
RLU / ug of protein
250
200
150
100
50
0
Control
Vatalanib
GKT + Vatalanib
NoxA1ds + Vatalanib
+Vatalanib
GKT + Vatalanib
NoxA1ds + Vatalanib
5 min
10 min


A
Nox4 mRNA
1.5
1.0
0.5
0.0
*
Control
8h
24h
Vatalanib
B
Nox5 mRNA
5
4
3
2
1
0
*
Control
8h
24h
Vatalanib
C
Catalase mRNA
1.5
1.0
0.5
0.0
*
*
Control
8h
24h
Vatalanib
D
GPX1 mRNA
1.5
1.0
0.5
0.0
*
Control
8h
24h
Vatalanib
E
HO1 mRNA
1.5
1.0
0.5
0.0
*
Control
8h
24h
Vatalanib
F
SOD1 mRNA
1.5
1.0
0.5
0.0
*
Control
8h
24h
Vatalanib


A
Relaxation (%)
0
40
80
120
Vehicle
Vatalanib
L-NAME
L-NAME + Vatalanib
φ
*
9
8
7
6
5
- Log [M] ACh
B
Relaxation (%)
Vehicle
Vatalanib
NAC
NAC + Vatalanib
*#
- Log [M] ACh
Veh
Vat 5 min
Veh
Vat 15 min
p-MLC20 (Thr18/Ser19) →
[18 kDa]
α-tubulin →
[50 kDa]
C
Vehicle
Vatalanib
NAC + Vatalanib
Contraction (mN)
10
8
6
4
2
0
*#
10
9
8
7
6
- Log [M] U46619
D
p-MLC20 / α-tubulin
250
200
150
100
50
0
Control
Vatalanib 5 min
Vatalanib 15 min
E
Max - Min - Percentage of baseline (Ex490 / Em535)
300
250
200
150
100
Vatalanib
NAC + Vatalanib
Vatalanib
0
30
60
90
120
150
180
Time (s)
Area under curve
25000
20000
15000
10000
5000
0
#
Vatalanib
NAC+Vatalanib


A
Vehicle
Vatalanib
Gefitinib
120
80
40
0
E_{max} ACh
*
Vehicle
Vatalanib
Gefitinib
B
120
80
40
0
E_{max} SNP
Vehicle
Vatalanib
Gefitinib
C
150
100
50
0
H_2O_2 production / protein (μM / μg - % control)
Aorta
Heart
**
**
Vehicle
Vatalanib
Gefitinib
Vehicle
Vatalanib
Gefitinib
D
80
60
40
20
0
Nitrotyrosine levels / Protein (μg)
Aorta
Heart
*
*
*
Vehicle
Vatalanib
Gefitinib
Vehicle
Vatalanib
Gefitinib
E
150
100
50
0
Catalase Activity / Protein (μg) (% Control)
Aorta
Heart
**
**
Vehicle
Vatalanib
Gefitinib
Vehicle
Vatalanib
Gefitinib


A
Kidney
RLU / ug of protein
300
200
100
0
*
Vehicle
Vatalanib
Gefitinib
B
H2O2 production / protein
(µM / µg - % control)
400
300
200
100
0
*
Vehicle
Vatalanib
Gefitinib
C
Catalase Activity / protein (µg)
(% Control)
150
100
50
0
*
Vehicle
Vatalanib
Gefitinib
D
Nrf2 Nuclear Translocation
[Arbitrary Units]
150
100
50
0
***
***
Vehicle
Vatalanib
Gefitinib
E
Keap-1 (Cytosol) →
(70 kDa)
α-Tubulin →
(50 kDa)
Keap-1 / α-tubulin
200
150
100
50
0
*
Vehicle
Vatalanib
Gefitinib
F
2ΔΔCt
15
10
5
0
Catalase
GPX1
*
*
*
*
*
Vehicle
Vatalanib
Gefitinib
Vehicle
Vatalanib
Gefitinib


VEGF
VEGFR
Vatalanib
Nox1/4
?
Ca^{2+}
↑ ROS
VSMC
O_2^-
↑ p-MLC
↑ Redox Signaling
Ca^{2+} Signaling
↑ Contraction
VEGF
Nox4
VEGFR
Vatalanib
Nox5
(-)
(+)
Nrf2
↑ ROS
↓ H_2O_2
↓ SOD1
Catalase
GPX1
↓ p-eNOS
O_2^-
↑ ONOO⁻
↓ NO
Endothelial
Dysfunction
EC
Hypertension