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

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25 **ABSTRACT**

26 Although vascular endothelial growth factor (VEGF) inhibitors (VEGFIs), are effective anti-
27 cancer therapies, they cause hypertension through unknown mechanisms. We questioned
28 whether changes in vascular redox state may be important, since VEGF signaling involves nitric
29 oxide (NO) and reactive oxygen species (ROS). Molecular mechanisms, including NOS, Nox-
30 derived ROS, anti-oxidant systems and vasoconstrictor signaling pathways, were probed in
31 human endothelial cells (EC) and vascular smooth muscle (hVSMC) exposed to vatalanib, a
32 VEGFI. Vascular functional effects of VEGFI were assessed *ex vivo* in mouse arteries.
33 Cardiovascular and renal *in vivo* effects were studied in vatalanib- or gefitinib (epidermal growth
34 factor inhibitor (EGFI))-treated mice. In ECs, vatalanib decreased eNOS (Ser¹¹⁷⁷)
35 phosphorylation and reduced NO and H₂O₂ production, responses associated with increased
36 Nox-derived O₂⁻ and ONOO⁻ formation. Inhibition of Nox1/4(GKT137831) or Nox1 (NoxA1ds),
37 prevented vatalanib-induced effects. Nrf2 nuclear translocation and expression of Nrf-2-
38 regulated anti-oxidant enzymes were variably downregulated by vatalanib. In hVSMCs, VEGFI
39 increased Nox activity and stimulated Ca²⁺ influx and MLC₂₀ phosphorylation. Acetylcholine-
40 induced vasodilatation was impaired and U46619-induced vasoconstriction was enhanced by
41 vatalanib, effects normalized by N-acetyl-cysteine and worsened by L-NAME. In vatalanib-, but
42 not gefitinib-treated mice vasorelaxation was reduced and media:lumen ratio of mesenteric
43 arteries was increased with associated increased cardiovascular and renal oxidative stress,
44 decreased Nrf-2 activity and downregulation of anti-oxidant genes. We demonstrate that
45 inhibition of VEGF signaling induces vascular dysfunction through redox-sensitive processes.
46 Our findings identify Noxs and antioxidant enzymes as novel targets underling VEGFI-induced

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

49

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

52

53 **INTRODUCTION**

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

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

70 reporting an increase in blood pressure (BP) as an adverse effect with 40-60% of patients
71 developing hypertension often severe (>150/100 mmHg) or hypertensive crisis⁹⁻¹². Hypertension
72 develops acutely, within 24 hours of starting treatment, and by 6 days it is sustained¹³. Upon
73 treatment cessation BP decreases^{9,10,14}.

74 The pathophysiology of VEGFI-induced hypertension is elusive, although endothelial
75 dysfunction, vascular remodeling and capillary rarefaction have been implicated^{2,15}. VEGF is a
76 known vasodilator through its effects on NO and as such a potential consequence of VEGFI is
77 reduced NO, impaired vasodilatation and increased vascular tone, important determinants of
78 augmented vascular resistance and BP elevation¹⁶⁻¹⁸. However clinical and experimental data are
79 conflicting, with studies showing both increased and decreased eNOS activity and NO
80 production^{16,17}. Additionally, recent findings demonstrated endothelial-independent processes
81 are involved in VEGFI effects¹⁹. Increased levels of ET-1, activation of the renin-angiotensin
82 system (RAS), EC apoptosis and rarefaction have also been implicated in VEGFI-induced
83 hypertension^{17,18}.

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

93 antioxidants, may influence vasorelaxation. However, whether VEGFI impacts these redox-
94 sensitive processes to modulate a hypertensive vascular phenotype with associated BP elevation
95 is unclear. Here we hypothesized that VEGFIs promote oxidative stress leading to impaired
96 vasodilatation and hypercontractility, processes associated with BP elevation. Mechanisms
97 underlying this may relate to Nox dysregulation, downregulation of Nrf-2 -regulated antioxidant
98 systems and altered Ca²⁺ handling in vascular cells.

99

100 **METHODS**

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

103

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

111 **Experimental models**

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

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

118

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

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

127 **Experimental protocols**

128 Vascular levels of NO, O₂⁻, H₂O₂ and nitrotyrosine and Nox activity were assessed by
129 fluorescence, chemiluminescence and amplex red assays. VSMC [Ca²⁺]_i was measured by Cal-
130 520 fluorescence.

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

134 Phosphorylation of eNOS and MLC20 was determined by immunoblotting.

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

136 **Statistical analysis**

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

142 **RESULTS**

143 **Vatalanib influences ROS and NO generation in human endothelial cells**

144 Vatalanib increased NADPH-dependent O_2^- generation in HAECs, effects that were inhibited by
145 GKT137831 and NoxA1dstat (**figure 1A**). This was associated with increased p47phox
146 membrane expression (**figure 1B**), reduced generation of H_2O_2 and NO and increased formation
147 of ONOO⁻ (**figures 1C, 1D, S1**). In vatalanib-treated cells, phosphorylation of eNOS (active site,
148 Ser¹¹⁷⁷) was reduced (**figure S1**). Vatalanib, at 24 hours, influenced expression of endothelial
149 cell Noxs, which are responsible for O_2^- production. In particular vatalanib decreased expression
150 of Nox4 and increased expression of Nox5 (**figure 2A, 2B**), without significantly influencing
151 Nox1 (**figure S2**). Nuclear accumulation of Nrf2 and gene expression of Nrf-2-regulated anti-
152 oxidant genes, catalase, GPX1 and HO1, but not SOD1, was downregulated 8 hours after
153 vatalanib treatment (**figure 1E, 2C-F**). Gefitinib does not alter O_2^- production neither modulates
154 Noxs and anti-oxidants mRNA levels in vascular cells (**figure S6**).

155 **Vatalanib increases ROS generation and modulates pro-contractile signaling in human** 156 **vascular smooth muscle cells**

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

159 significant increase in Ca^{2+} influx in hVSMCs, effects that were attenuated by N-acetyl-l-
160 cysteine (NAC) (**figure 3E**). Vatalanib also influenced pro-contractile signaling pathways, by
161 inducing phosphorylation of MLC_{20} , critically involved in triggering vascular contraction (**figure**
162 **3D**).

163 **Vascular dysfunction induced by vatalanib is mediated by NOS- and redox-sensitive** 164 **mechanisms**

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

175 **Systemic oxidative stress, vascular dysfunction and arterial remodeling in vatalanib-** 176 **treated mice.**

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

181 (88.0±2.5 mmhg). Vatalanib increased systemic ROS generation, as indicated by elevated
182 plasma TBARS levels in the vatalanib (9.0±2.0 μmol/l) versus vehicle (5.1±0.2 μmol/l) and
183 gefitinib groups (5.2±0.5 μmol/l).

184 As shown in **figure 4A** and supplemental table S1, ACh-induced maximal vasorelaxation
185 and EC₅₀ of isolated small mesenteric arteries were blunted in vatalanib- but not gefitinib-treated
186 mice. SNP-induced vasodilatation was not influenced by either agent (**figure 4B**). Mesenteric
187 arteries from vatalanib-treated mice also exhibited an increase in media-to-lumen ratio indicating
188 vascular remodeling (**figure S3**). Vatalanib had no effect on cross-sectional area (CSA) (**figure**
189 **S3**). Gefitinib did not significantly influence vascular function or structure.

190 **Vatalanib modulates redox signaling in tissues from mice**

191 To determine whether VEGFIs influence redox status in cardiovascular and renal tissue, we
192 assessed levels of H₂O₂, O₂⁻ and ONOO⁻, catalase activity and expression of pro-oxidant
193 oxidases and anti-oxidant enzymes in aorta, kidney and heart in VEGFI- treated mice.

194 As shown in figure 4 aortic and cardiac levels of H₂O₂ were reduced, while ONOO⁻
195 levels were increased. Catalase activity was increased in aorta in the vatalanib group. Gene
196 expression of Nox1, but not Nox2 or Nox4, was significantly increased in the heart by vatalanib
197 and gefitinib (**figure S4**). Cardiac gene expression of anti-oxidant enzymes catalase and GPX1
198 was reduced in vatalanib-treated mice, without effect on SOD1 (**figure S4**).

199 NADPH-stimulated production of O₂⁻ and H₂O₂ levels were augmented by vatalanib in
200 kidneys (**figures 5A, 5B**). This was associated with decreased activity of renal catalase (**figure**
201 **5C**) and downregulation of the master anti-oxidant transcription factor Nrf2, indicated by
202 decreased Nrf2 nuclear translocation and increased cytosolic levels of the Nrf2 repressor, Keap-1
203 (**figures 5D, 5E**). At the gene level, expression of anti-oxidant enzymes catalase and GPX1

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

206

207 DISCUSSION

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

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

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

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

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

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

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

273 To elucidate whether inhibition of VEGFR tyrosine kinases by vatalanib is a generalized
274 or specific phenomenon, we also evaluated effects of gefitinib, which inhibits EGFR, in part,
275 through common VEGFR signaling pathways. Our findings clearly demonstrate that vatalanib,
276 but not gefitinib induced vascular dysfunction and cardiovascular and renal oxidative stress,
277 suggesting specific cardiovascular toxicity when VEGFR is targeted. Others have also shown
278 differential effects of VEGFI and EGFI^{28,29}. Mice treated with sunitinib, which primarily
279 targets VEGFR tyrosine kinases, exhibited systolic dysfunction and metabolic abnormalities,
280 which were absent in mice treated with the EGFR I erlotinib⁴⁰. Using a non-targeted
281 metabolomics approach, it was found that sunitinib, but not erlotinib, decreased docosahexaenoic
282 acid (DHA), arachidonic acid (AA)/ eicosapentaenoic acid (EPA), O-phosphocolamine, and 6-
283 hydroxynicotinic acid, important anti-inflammatory mediators and regulators of mitochondrial
284 function. Loss of these compounds may underlie VEGFI-induced mitochondrial dysfunction,
285 oxidative stress and cardiovascular damage⁴⁰.

286 Despite the vascular dysfunction, arterial remodelling and significant cardiovascular and
287 renal oxidative effects induced by vatalanib, mice did not develop hypertension. Reasons for this
288 may relate to the low dose of vatalanib used and/or to the relatively short treatment period. In
289 addition, we used tail cuff methodology to measure blood pressure at one time point, and as such
290 we may have missed subtle changes in blood pressure, especially over the 24-hour period.
291 Telemetry would have provided a better approach to fully characterise blood pressure changes.
292 Nevertheless, our data clearly demonstrate, that even at sub-pressor doses, vatalanib induced
293 cardiovascular and renal toxicity, which may be amplified with higher doses and more chronic
294 treatment³⁹.

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

305 **PERSPECTIVES**

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

315

316

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322

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325

326 **DISCLOSURES**

327 None.

328

329 **REFERENCES**

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464 **NOVELTY AND SIGNIFICANCE**

465 **What Is New?**

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

469 **What Is Relevant?**

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

477

478 **Summary**

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

485

486

487 **FIGURE LEGENDS**

488 **Figure 1. Vatalanib influences ROS and NO generation and modulates pro-contractile**
489 **signaling in human vascular cells.** (A) Lucigenin-derived chemiluminescence was performed
490 in HAEC stimulated with vehicle or vatalanib in the presence or absence of NoxA1ds or
491 GKT137831. (B) p47 phox membrane expression was assessed by western blotting in HAEC in
492 presence of vatalanib or vehicle. (C) H₂O₂ levels, measured by amplex red in HAEC exposed to
493 vatalanib. (D) NO levels were determined by DAF-FM in vatalanib-treated HAEC (5 minutes) in
494 the presence or absence of GKT137831 or NoxA1ds (30 minutes). (E) Nuclear accumulation of
495 Nrf2 was determined by ELISA in HAEC nuclear extracts. (F) Lucigenin-derived
496 chemiluminescence was performed in hVSMC stimulated with vehicle or vatalanib±NoxA1ds or
497 GKT137831. Results from amplex red, lucigenin and DAF-FM assays were normalized by
498 protein content. Results are means±SEM of 4 to 7 experiments. *p<0.05 vs. control; # vs.
499 vatalanib.

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

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

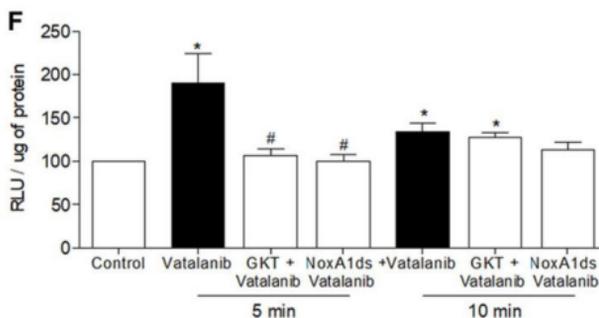
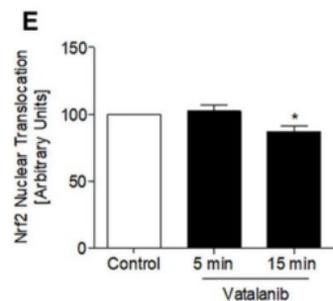
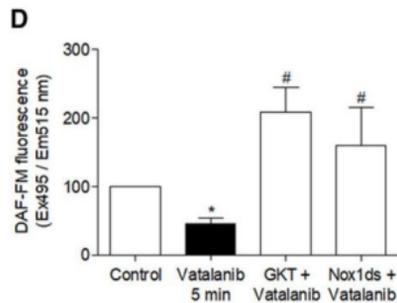
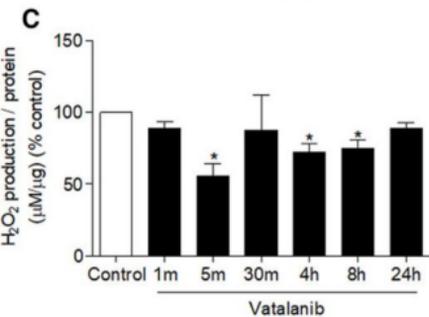
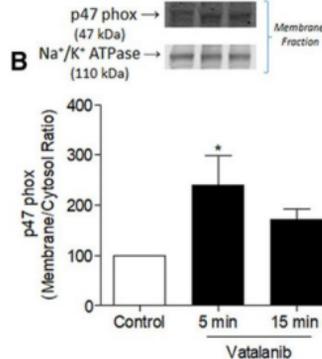
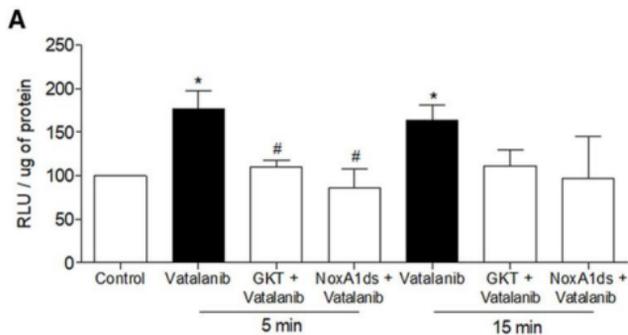
510 ROS scavenger, 10 $\mu\text{mol/L}$) was added 30 min before vehicle or vatalanib addition. (C)
511 Contraction curves to U46619 in the absence or presence of NAC. (D) Phosphorylation of
512 MLC_{20} ($\text{Thr}^{18}/\text{Ser}^{19}$) was determined by immunoblotting in hVSMC stimulated with vatalanib
513 (100 nmol/L); values were normalized by α -tubulin expression. (E) Ca^{2+} influx was assessed in
514 hVSMC exposed to vatalanib \pm NAC. Area under the curve (AUC) indicates global Ca^{2+} influx
515 in hVSMC. Results are mean \pm SEM of 3-6 experiments. * $p < 0.05$ vs. vehicle; # $p < 0.05$ vs.
516 vatalanib; ω $p < 0.05$ vs. L-NAME.

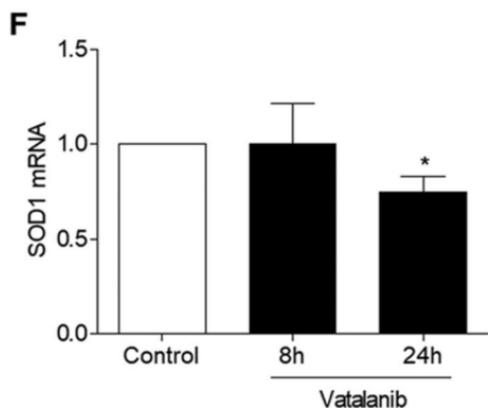
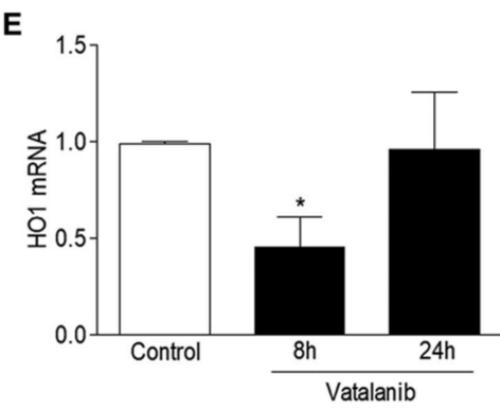
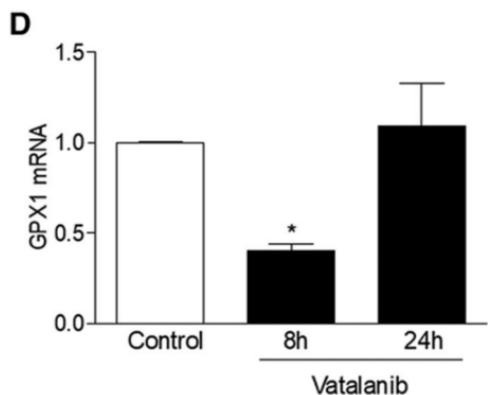
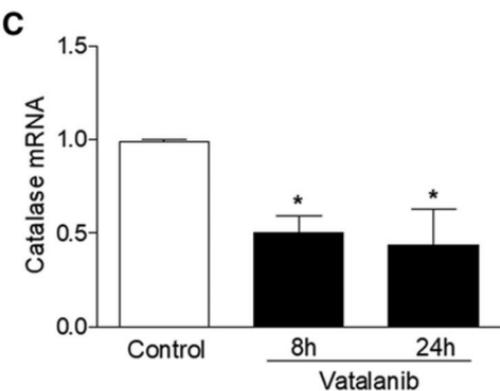
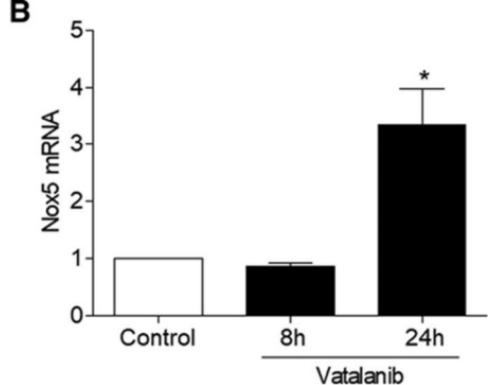
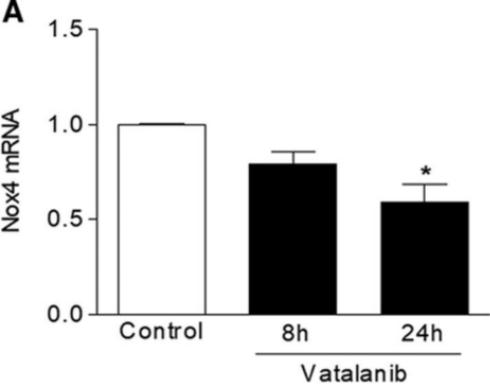
517 **Figure 4. Vatalanib, but not gefitinib, impairs ACh-induced vasodilation and modulates**
518 **redox signaling in aorta and heart from mice.** Bar graphs represent the maximal response
519 (E_{max}) of ACh (A) and SNP (B) in mesenteric arteries from mice treated with vatalanib (100
520 mg/Kg/day),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

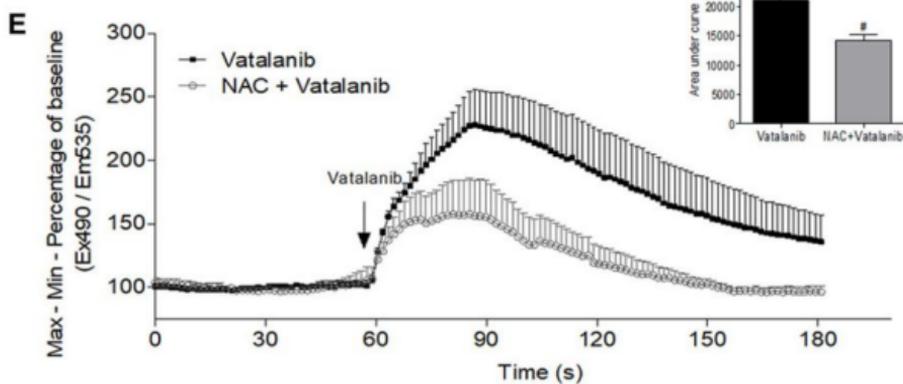
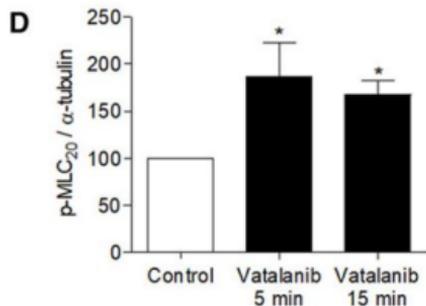
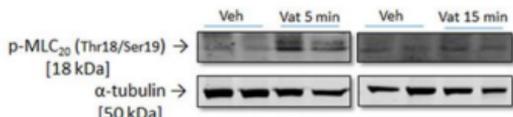
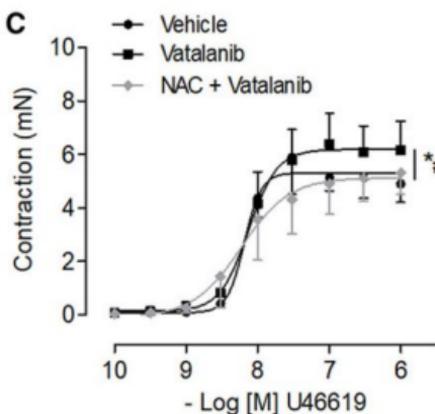
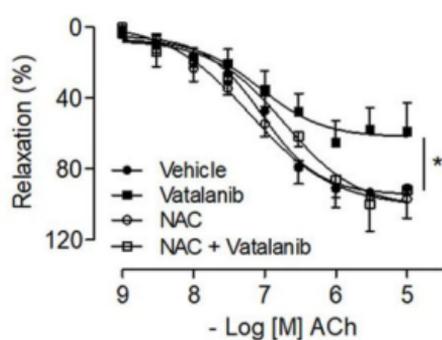
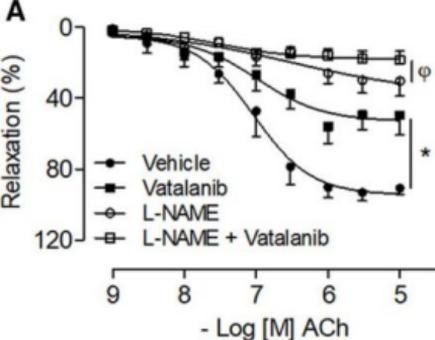
526 **Figure 5. Renal oxidative and anti-oxidant effects of vatalanib.** (A) NADPH-stimulated O_2^-
527 production, assessed by lucigenin assay, in kidneys from vatalanib and gefitinib-treated mice.
528 (B) H_2O_2 levels and catalase activity (C) measured by amplex red assay in kidneys. (D) Nuclear
529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

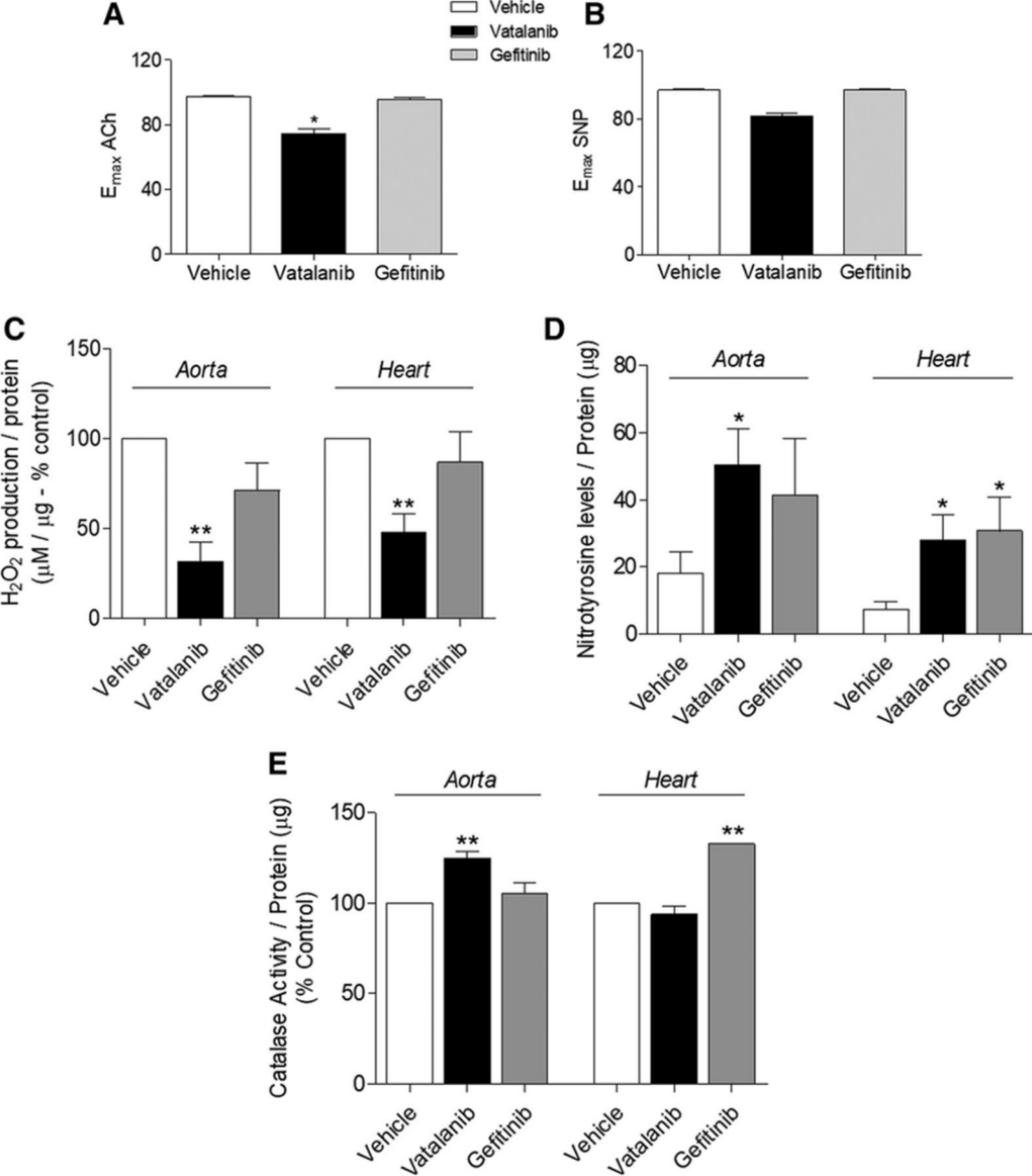
532 normalized by GAPDH mRNA expression and Keap-1 values were normalized by α -tubulin
533 protein expression. Results are mean \pm SEM of 5-7 experiments. *p<0.05 vs. vehicle.

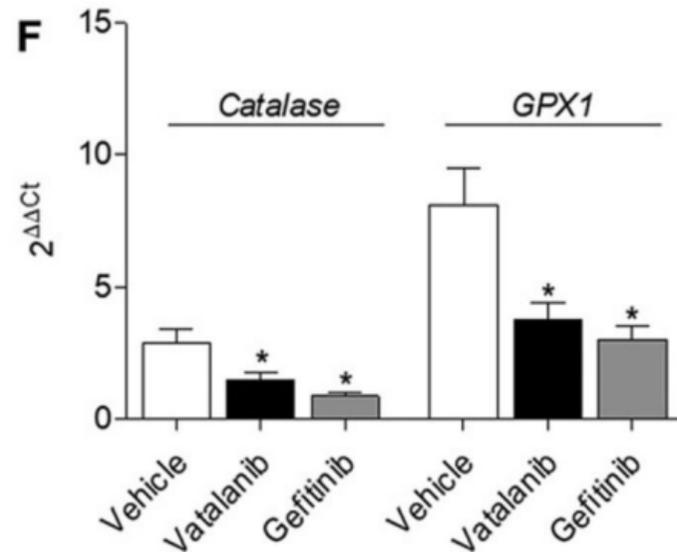
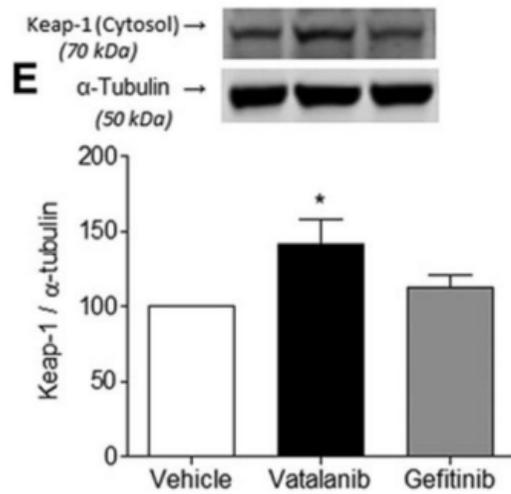
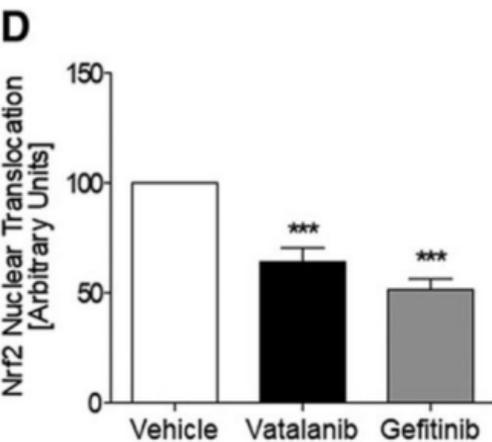
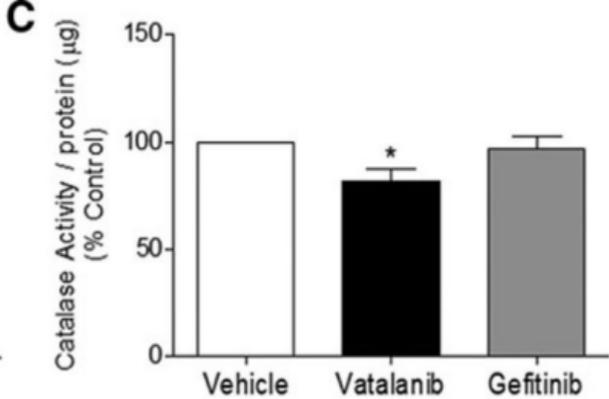
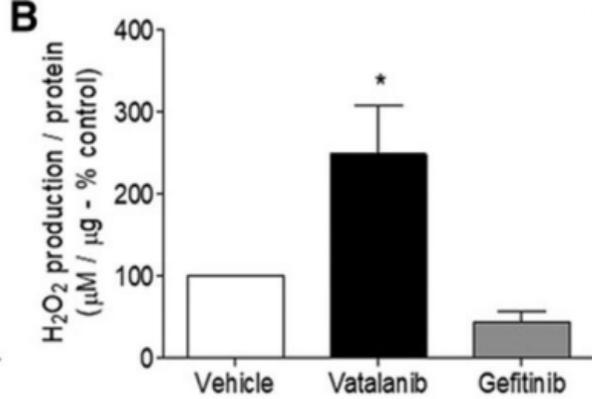
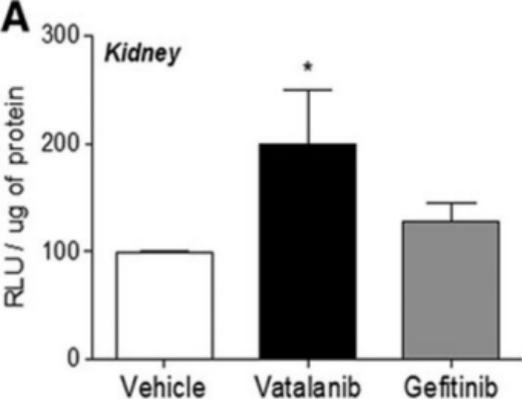
534 **Figure 6. Putative mechanisms involving redox-sensitive processes whereby VEGF/VEGFR**
535 **inhibition impacts vascular smooth muscle and endothelial cell function.** VEGF inhibition
536 by vatalanib in hVSMC (left panel) causes an increase in ROS generation through Nox1 and 4
537 activation which is involved in pro-contractile signaling, such as increased $[Ca^{2+}]_i$ and activation
538 of MLC₂₀, leading to enhanced vascular contraction. In endothelial cells (right panel), vatalanib
539 acts by increasing ROS production through Nox activation but also by downregulating the
540 antioxidant system. In addition, vatalanib decreases the vasodilatory ROS, H₂O₂, as well as
541 reduces eNOS phosphorylation and NO production in endothelial cells, which may be
542 culminating in endothelial dysfunction. The dysregulation of both vascular smooth muscle and
543 endothelial cell function induced by vatalanib may induce vascular tone alterations and vascular
544 remodeling, and may explain, at least in part, molecular mechanisms underlying VEGFI-
545 associated hypertension.

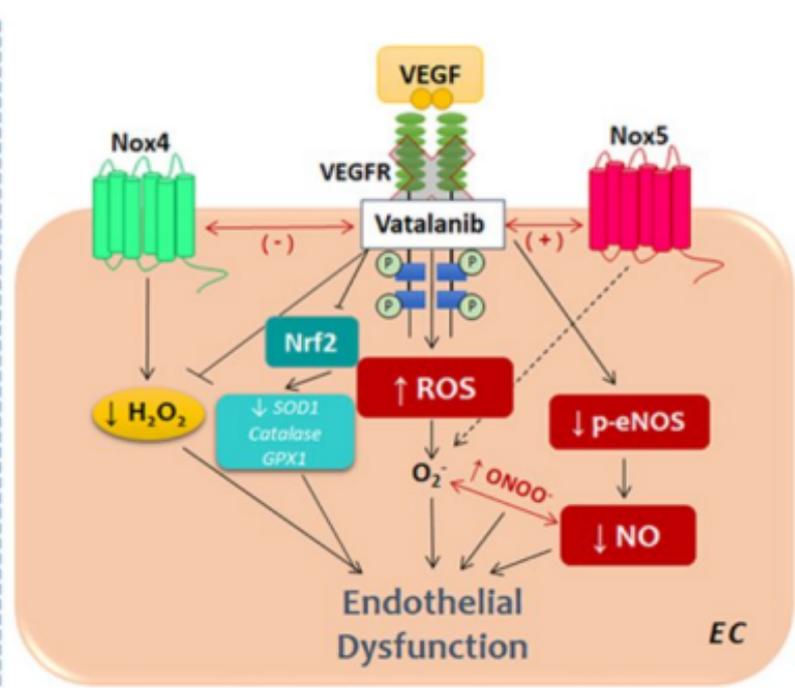
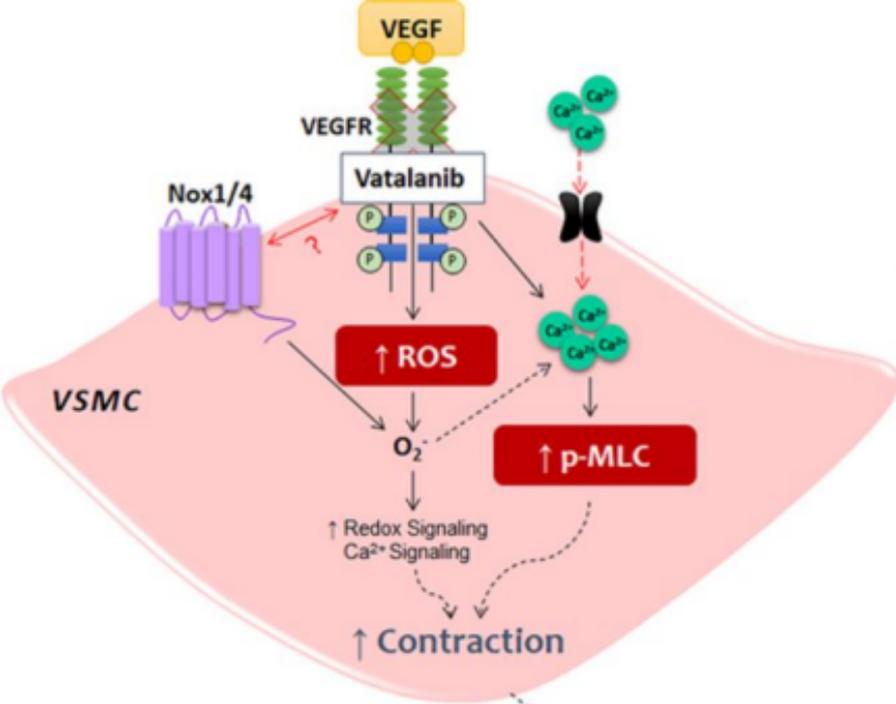












Hypertension