



## ORIGINAL RESEARCH COMMUNICATION

# Direct Activation of NADPH Oxidase 2 by 2-Deoxyribose-1-Phosphate Triggers Nuclear Factor Kappa B-Dependent Angiogenesis

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### Abstract

**Aims:** Deoxyribose-1-phosphate (dRP) is a proangiogenic paracrine stimulus released by cancer cells, platelets, and macrophages and acting on endothelial cells. The objective of this study was to clarify how dRP stimulates angiogenic responses in human endothelial cells.

**Results:** Live cell imaging, electron paramagnetic resonance, pull-down of dRP-interacting proteins, followed by immunoblotting, gene silencing of different NADPH oxidases (NOXs), and their regulatory cosubunits by small interfering RNA (siRNA) transfection, and experiments with inhibitors of the sugar transporter glucose transporter 1 (GLUT1) were utilized to demonstrate that dRP acts intracellularly by directly activating the endothelial NOX2 complex, but not NOX4. Increased reactive oxygen species generation in response to NOX2 activity leads to redox-dependent activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B), which, in turn, induces vascular endothelial growth factor receptor 2 (VEGFR2) upregulation. Using endothelial tube formation assays, gene silencing by siRNA, and antibody-based receptor inhibition, we demonstrate that the activation of NF- $\kappa$ B and VEGFR2 is necessary for the angiogenic responses elicited by dRP. The upregulation of VEGFR2 and NOX2-dependent stimulation of angiogenesis by dRP were confirmed in excisional wound and Matrigel plug vascularization assays *in vivo* using NOX2<sup>-/-</sup> mice.

**Innovation:** For the first time, we demonstrate that dRP acts intracellularly and stimulates superoxide anion generation by direct binding and activation of the NOX2 enzymatic complex.

**Conclusions:** This study describes a novel molecular mechanism underlying the proangiogenic activity of dRP, which involves the sequential activation of NOX2 and NF- $\kappa$ B and upregulation of VEGFR2. *Antioxid. Redox Signal.* 28, 110–130.

**Keywords:** angiogenesis, endothelial, NADPH, NF- $\kappa$ B, NOX, ROS

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### Innovation

A novel paracrine signaling pathway is described in this study, whereby the angiogenic stimulus 2-deoxyribose-1-phosphate (dRP) released by platelets, macrophages, and cancer cells is internalized by endothelial cells and acts intracellularly by directly activating NADPH oxidase 2 (NOX2). In turn, this leads to nuclear factor kappa B (NF- $\kappa$ B)-dependent upregulation of vascular endothelial growth factor receptor 2 (VEGFR2) and VEGF-dependent angiogenesis. Our elucidation of the mechanism of action of dRP is critical for understanding the full potential of this proangiogenic paracrine signal, which may find important applications in regenerative, vascular, and cancer medicine.

### Introduction

ANGIOGENESIS IS CRITICAL for tissue revascularization and repair after injury. Reactive oxygen species (ROS) have been shown to drive tissue repair by triggering angiogenesis (32). The most important of such factors is vascular endothelial growth factor (VEGF), which is upregulated as a consequence of hypoxia-induced factor-1 (HIF-1) activation (1, 28). The increase in ROS triggered by hypoxia and other tissue injuries is associated with oxidation of biological molecules such as lipids and proteins, which has profound effects on cellular physiology (23). Among the sources of ROS associated with the stimulation of angiogenesis, NADPH oxidases (or NOXs) have been studied extensively (65, 69). The precise links between NOX activation and angiogenesis remain unclear, but several molecular mechanisms have been implicated, including activation of nitric oxide synthase (13) and VEGF upregulation (32, 69). Endothelial cells express NOX1, NOX2, NOX4, and NOX5 (18). Among NOXs, NOX4 has been shown to lead to stabilization of HIF-1, which in turn stimulates increased transcription of VEGF and drives angiogenesis (69). Other factors, including insulin and TGF- $\beta$ 1, also stimulate angiogenesis in an NOX4-dependent manner (41, 46). Similarly

to NOX4, NOX2 is abundantly expressed in endothelial cells and mediates angiogenesis in response to lipopolysaccharide (LPS) and VEGF (19, 40). In keeping with their roles as positive regulators of angiogenesis, both NOX2 and NOX4 can drive endothelial cell migration and capillary-like tube formation in hyperoxic conditions (45). In addition to their effects on the HIF/VEGF signaling axis, NOXs have been shown to stimulate angiogenesis through diverse signaling pathways. For example, activation of nuclear factor kappa B (NF- $\kappa$ B) downstream of NOX2 (39) or nuclear factor erythroid 2-related factor 2 (Nrf-2) downstream of NOX4 (57) has been shown to be involved in stimulating the angiogenic responses of endothelial cells.

Deoxyribose-1-phosphate (dRP) has previously been described as an endogenous molecule capable of stimulating angiogenesis in an ROS-dependent manner both *in vitro* and *in vivo* (7, 24, 42, 49, 50, 58). The generation of dRP in eukaryotic cells is catalyzed by phosphorylases with specificity for different nucleosides. Three main enzymes have been characterized: thymidine phosphorylase (TP), uridine phosphorylase (UP), and purine nucleoside phosphorylase (PNP) (48). Nucleoside phosphorylases play a key role in nucleoside and pentose metabolism by degrading nucleosides into free nitrogen base and dRP, with dRP converted to deoxyribose-5-phosphate by phosphopentomutase (64). Several studies have suggested that nucleoside phosphorylases stimulate cancer angiogenesis in solid tumors and participate in the progression of the disease (27, 31, 62). Although regulation of nucleoside phosphorylases is largely unknown and their constitutive activity has been described (5), we previously presented data on the release of dRP by human platelets in response to cellular stimulation (67). In this study, we have evaluated the proangiogenic activity of dRP on human umbilical vein endothelial cells (HUVECs) *in vitro* using a variety of molecular techniques and have identified the NOX2-NF- $\kappa$ B signaling axis that is engaged by dRP, resulting in the upregulation of VEGF receptor 2 (VEGFR2) expression and stimulation of angiogenic responses. This study is the most comprehensive and exhaustive characterization of dRP as a proangiogenic stimulus to date.

**FIG. 1. dRP stimulates angiogenesis and oxidative stress of endothelial cells *in vitro*.** (A) The concentration of dRP released by human platelets and mouse macrophages *in vitro* was quantified by LC-MS. Presented data are from six and three independent samples, respectively. Statistical significance was assessed by one-way ANOVA with Bonferroni *post hoc* test (\* $p$  < 0.01 compared with nonstimulated platelets). (B) HUVECs were seeded at a density of  $3 \times 10^2$  cells/mm<sup>2</sup> on growth factor-reduced Matrigel<sup>®</sup> and cultured in basal medium (no FBS). Different concentrations of dRP between 2  $\mu$ M and 1 mM and after 4 h of culture and quantification of tube number per optical field were performed using the Angiogenesis Analyzer plug-in of ImageJ. (C) ROS generation was analyzed with DHE staining for 1 h in response to concentrations of dRP ranging from 2  $\mu$ M to 1 mM and expressed as fold increase over basal level. (D) Time course of ROS generation in response to 200  $\mu$ M dRP in the presence of ROS scavengers (1 mM NAC, 10  $\mu$ M MnTBAP, or 10  $\mu$ M Tempol) or vehicle. ROS production was assessed after 5, 30, 60, and 120 min and expressed as fold increase over basal level. (E) HUVECs were seeded at a density of  $3 \times 10^2$  cells/mm<sup>2</sup> on growth factor-reduced Matrigel and cultured in basal medium (no FBS). Two hundred micromolars of dRP was incubated in the presence or absence of 1 mM NAC, 10  $\mu$ M Tempol, or 10  $\mu$ M MnTBAP. After 4 h of culture, quantification of tube number per optical field was performed using the Angiogenesis Analyzer plug-in of ImageJ. Representative pictures are shown (i) and quantification is shown (ii). Throughout the figure, data are expressed as mean  $\pm$  SEM and analyzed by one-way ANOVA (B, C) ( $n$  = 6); (E) ( $n$  = 8); or two-way ANOVA (D) ( $n$  = 6). In either case, Bonferroni *post hoc* test was used to identify statistically significant differences between conditions; \* $p$  < 0.05 compared with vehicle, \*\* $p$  < 0.05 compared with dRP. (E) Bars = 300  $\mu$ m. ANOVA, analysis of variance; DHE, dihydroethidium; dRP, deoxyribose-1-phosphate; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; LC-MS, liquid chromatography–mass spectrometry; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; SEM, standard error of the mean; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

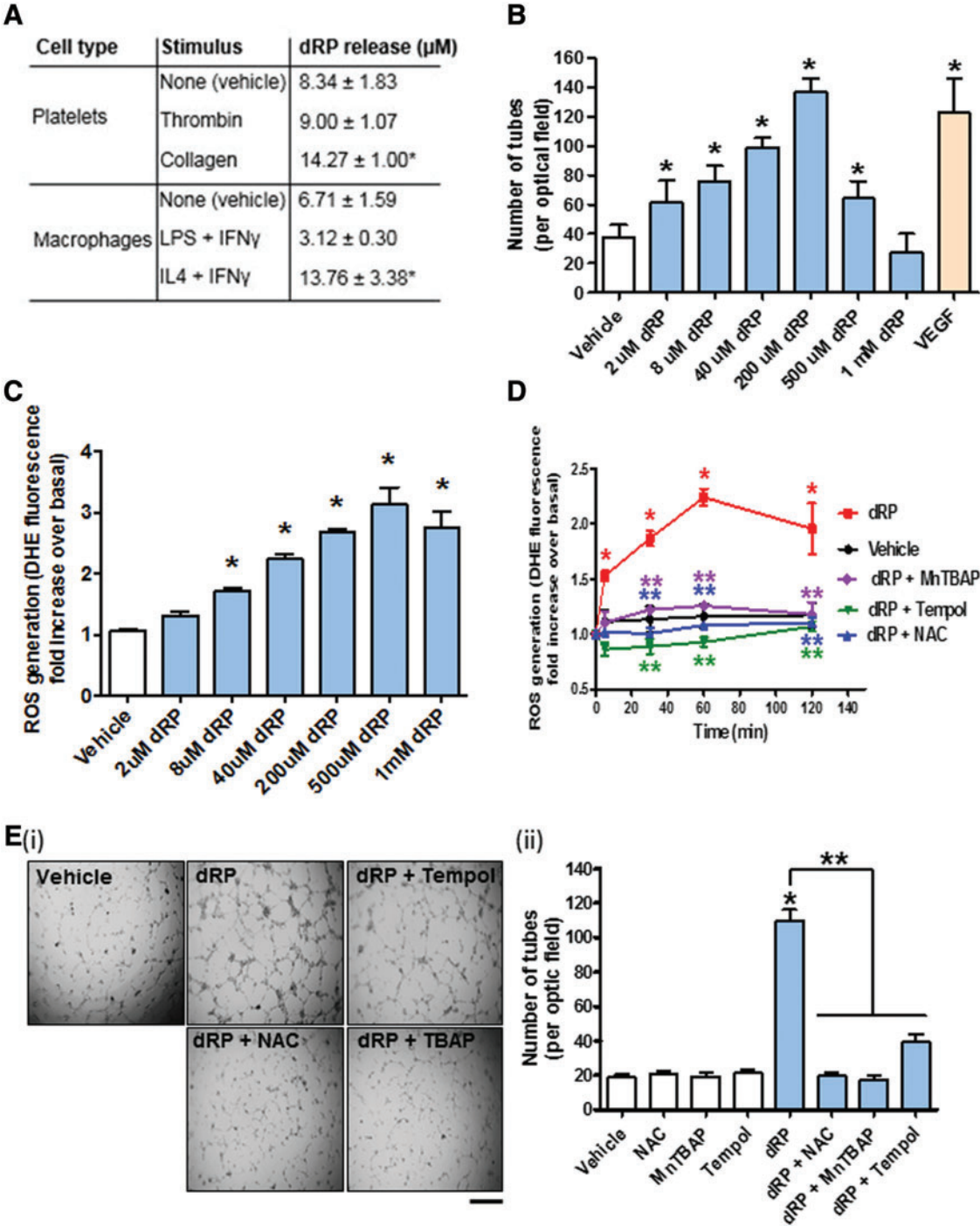
Understanding the molecular mechanisms underlying the actions of dRP as a proangiogenic stimulus will have important applications in cancer, vascular, and regenerative medicine.

Results

dRP stimulates increased levels of ROS generation in an NOX-dependent manner

We have previously described the release of dRP by human platelets (67). Using a quantitative liquid chromatography–mass spectrometry (LC-MS) method, we quantified dRP re-

leased by human platelets and mouse macrophages. In platelet suspensions at physiological density (*i.e.*,  $3 \times 10^8/\text{ml}$ ) and in culture medium from confluent murine macrophages, dRP reached concentrations above  $10 \mu\text{M}$  (Fig. 1A). The ability of dRP to induce the formation of capillary-like structures by endothelial cells *in vitro* (*i.e.*, endothelial tubes) was confirmed for concentrations as low as  $2 \mu\text{M}$  using low serum and growth factor-reduced Matrigel® (Fig. 1B and Supplementary Fig. S1A; Supplementary Data are available online at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)), while other pentoses were not effective (Supplementary Fig. S2). We also confirmed that



dRP concentrations as low as  $8\text{ }\mu\text{M}$  stimulate a significant increase in endothelial cell ROS formation (while  $2\text{ }\mu\text{M}$  dRP produced a trend toward increased ROS formation without reaching statistical significance), as measured using dihydroethidium (DHE) after 1 h of treatment (Fig. 1C). Complete time courses of ROS generation at low micromolar dRP concentrations are shown in Supplementary Figure S1B. The dRP-dependent increase in ROS generation rates was abolished in the presence of  $1\text{ mM}$  N-acetyl-L-cysteine (NAC),  $10\text{ }\mu\text{M}$  Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP), or  $10\text{ }\mu\text{M}$  4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) (Fig. 1D). The link between oxidative stress and angiogenic activity of HUVECs and the role of ROS generation in the angiogenic response induced by dRP were then tested using the ROS scavenger NAC (71) and the superoxide dismutase (SOD) mimetics, MnTBAP (21) and Tempol (33). All three significantly impaired the tubulogenic activity of dRP (Fig. 1E). Other angiogenic responses induced by dRP (*i.e.*, endothelial cell proliferation and monolayer scratch healing) were also inhibited by NAC, MnTBAP, and Tempol (Supplementary Fig. S3). Interestingly, the stimulatory effect of VEGF on endothelial tube formation (Supplementary Fig. S4) and monolayer scratch healing (Supplementary Fig. S5) was abolished by NOX inhibitors (*e.g.*, pan-NOX inhibitor VAS2870 and the NOX2-specific inhibitor peptide Nox2ds-tat), but not ROS scavengers (*e.g.*, NAC) and SOD mimetics (*e.g.*, MnTBAP).

Next, we tested the effect of dRP on redox homeostasis in HUVECs by electron paramagnetic resonance (EPR) using the superoxide-specific spin probe 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Fig. 2). In these experiments, we detected a significant increase in the superoxide anion generation rate in response to dRP from  $9.7 \pm 2.9$  to  $41.3 \pm 4.6\text{ pmol min}^{-1}\text{ mg}^{-1}$  of cell protein (mean  $\pm$  standard error of the mean; shown in Fig. 2A with calibration curve shown in Fig. 2C). Superoxide anion generation detected by EPR was inhibited by the SOD mimetic MnTBAP and by the pan-NOX inhibitor VAS2870 (Fig. 2B). The involvement of NOXs was also confirmed by coimmunoprecipitation of p47phox with NOX2 in the presence of dRP, but not in its absence (Fig. 2D).

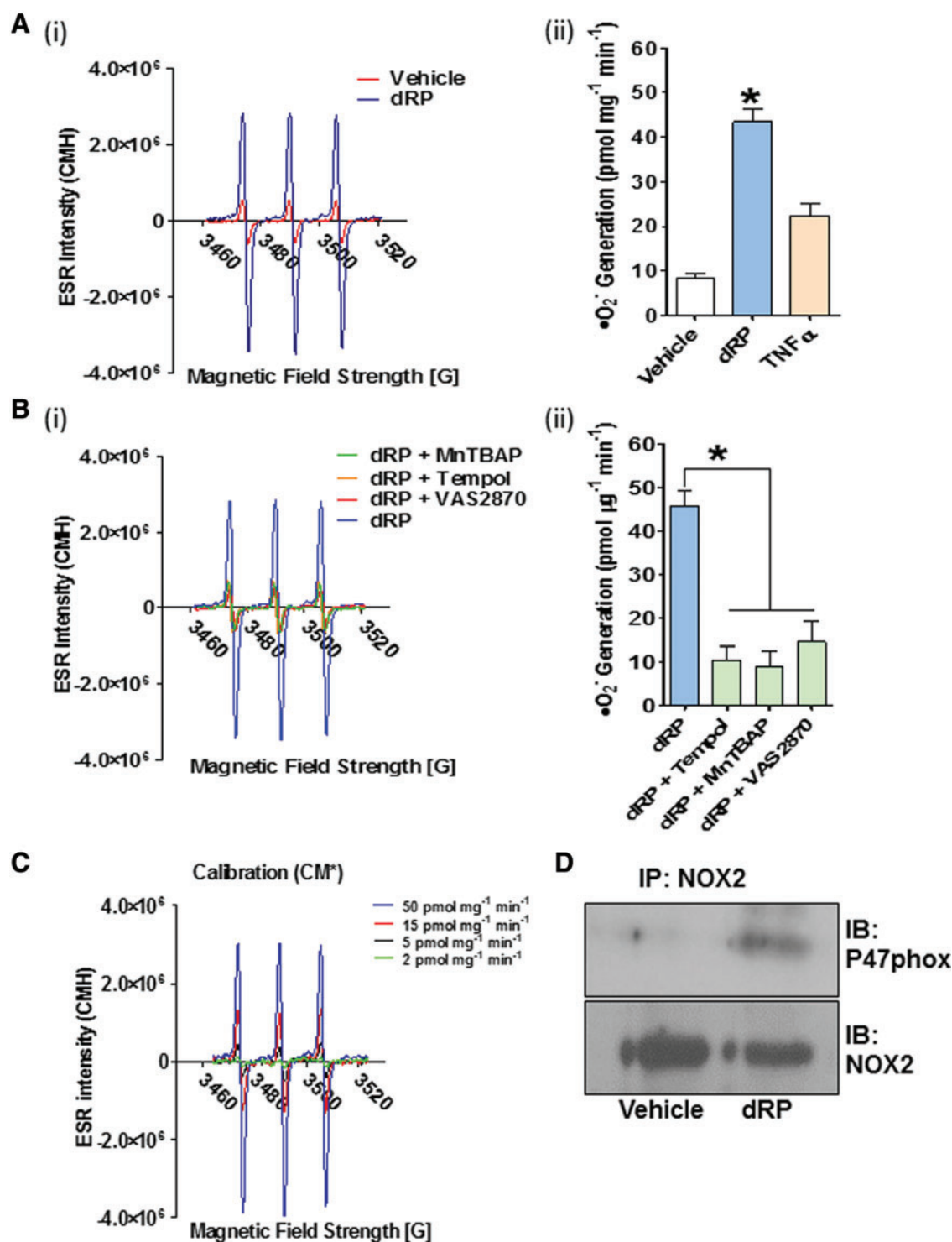
#### *dRP directly stimulates NOX2*

EPR analysis remains laborious and time consuming, which limits its application for large numbers of samples. Therefore, we also performed ROS generation analysis using live cell imaging with DHE, which confirmed dRP-induced ROS production in HUVECs. We then explored the source(s) of ROS generated in response to dRP. Treatment with the pan-NOX inhibitor VAS2870 ( $1\text{ }\mu\text{M}$ ) suppressed the dRP-induced increase in ROS generation (Fig. 3A). The specific inhibitory peptide Nox2ds-tat (54) was used to show that NOX2 is responsible for the stimulation of ROS generation by dRP. Treatment with Nox2ds-tat completely abolished the dRP-dependent increase in ROS generation in endothelial cells (Fig. 3B). Genetic silencing of NOX2 (*i.e.*, gp91phox subunit) also totally inhibited the dRP-dependent increase in the ROS generation rate (Fig. 3C) and endothelial tube formation (Fig. 3D). In contrast, genetic silencing of NOX4 did not significantly impair dRP-dependent ROS generation (Fig. 3E) or endothelial tube formation (Fig. 3F).

To test whether dRP acts intracellularly, we treated HUVECs for 30 min with  $200\text{ }\mu\text{M}$  dRP. Following cell disruption by ultracentrifugation, the cytoplasmic fractions were analyzed using LC-MS for the presence of dRP. Interestingly, dRP appeared in the cytoplasm of HUVECs after treatment (Fig. 4A). As dRP is administered as a salt, the counter ion cyclohexylammonium was utilized as a control and was found only in the culture media, never in cytoplasmic fractions (data not shown). This suggested the existence of a specific transporter for dRP. Previous studies indicated that the transporter, GLUT1, is the endothelial transporter for several monosaccharides besides hexoses, including riboses (60). To test the hypothesis that GLUT1 is responsible for the internalization of dRP, we utilized two specific inhibitors of this transporter: fasentin and STF-31 ( $10\text{ }\mu\text{M}$ ). These inhibitors abolished the ability of dRP to stimulate endothelial tube formation on Matrigel, without affecting VEGF-stimulated tube formation (Fig. 4B). GLUT1 was then silenced by small interfering RNA (siRNA), which resulted in significant inhibition of dRP-dependent ROS generation (Fig. 4C) and tube formation (Fig. 4D). These data suggest that GLUT1 plays a significant role in the internalization and proangiogenic activity of dRP. VEGF-dependent tube formation was not affected by depletion of GLUT1 (Fig. 4D).

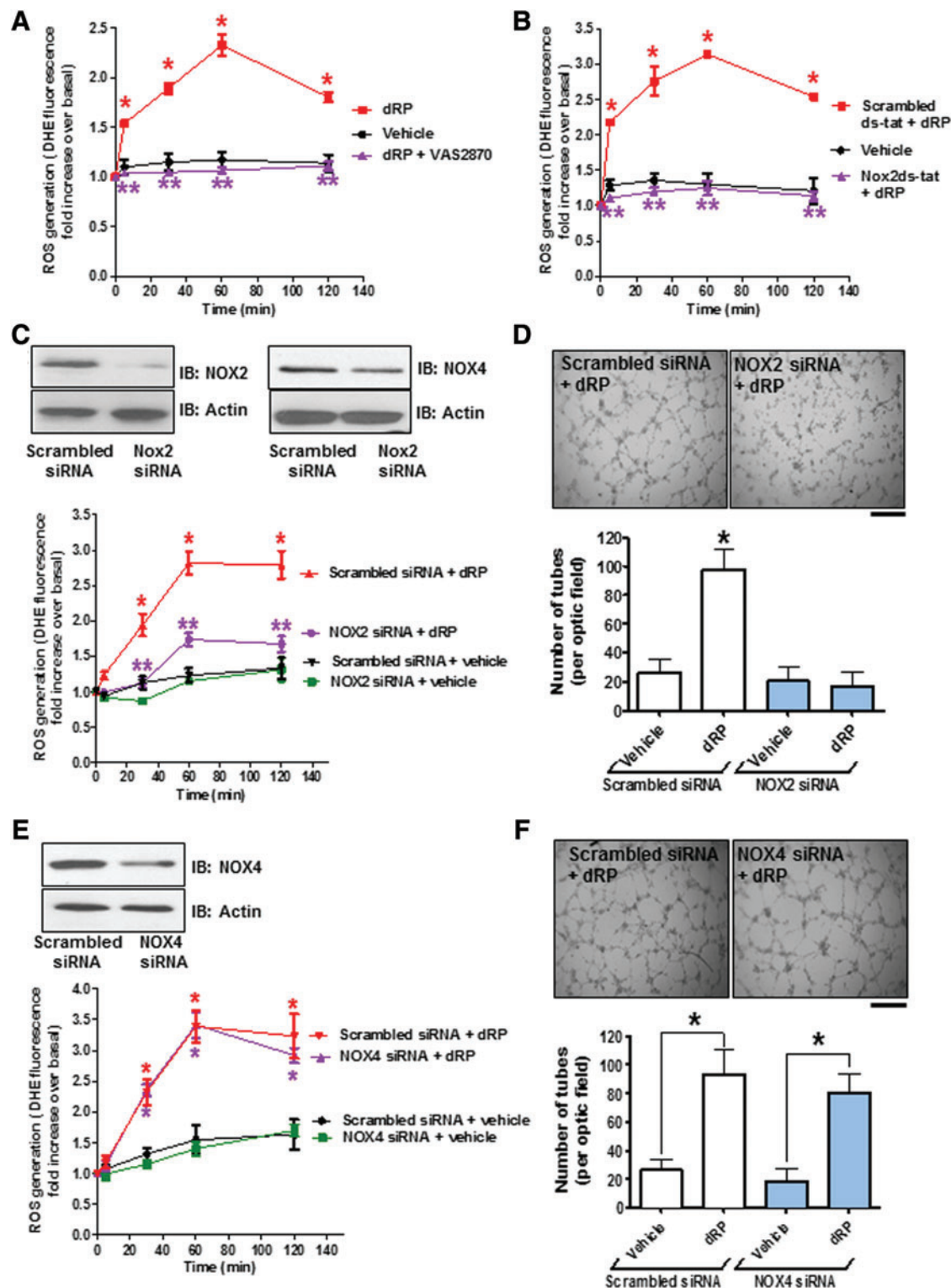
Direct binding of dRP to the NOX2 complex (but not to NOX4 or NOX1) was then demonstrated by pull-down experiments using biotinylated dRP and streptavidin beads. In these experiments, dRP was conjugated with biotin in a reaction leading to a mixture of three different adducts (C3-O-linked, C5-O-linked, and C1-OP-linked; Fig. 5A). NOX2 (but not NOX4 or NOX1) was selectively pulled down in the presence of biotinylated dRP, but not in the presence of nonbiotinylated dRP. The ability of dRP to activate NOXs directly without the mediation of intracellular signaling pathways was then investigated using a cell-free superoxide anion generation assay (9). Following cell fractionation, HUVEC cytosolic and membrane fractions were characterized for the expression of NOX1, NOX2, and NOX4. All three NOXs are expressed in the membrane fraction, but not in the cytoplasmic fraction (Fig. 5B). Caveolin-1 and actin were used as membrane and cytoplasmic markers, respectively. Upon treatment with dRP, HUVEC membrane fractions induced a significant increase in ferrous cytochrome c compared with membrane fractions treated with vehicle alone. The formation of ferrous cytochrome c was inhibited in the presence of SOD and by the nonspecific flavoenzyme inhibitor diphenylene iodonium (DPI). The dRP-dependent response registered in this assay was also inhibited by the highly specific peptide inhibitor Nox2ds-tat (54), which has been used for membrane-based cell-free assays previously (14). Taken together, these data suggest that dRP is able to directly activate NOX2 without mediation of other signaling events.

To confirm the oxidative status induced by dRP, biotinyl-iodoacetamide (BIAM) and horseradish peroxidase (HRP)-streptavidin were utilized to stain free thiols in HUVEC proteins. These experiments demonstrated that a 4-h exposure to  $200\text{ }\mu\text{M}$  dRP induces cysteine oxidation (*i.e.*, formation of sulfenic acid and/or disulfide bonds), which appears as a loss of BIAM staining (Fig. 6A). Several bands disappear or become significantly fainter upon treatment with dRP (green arrows in Fig. 6A), which suggests that this molecule induces

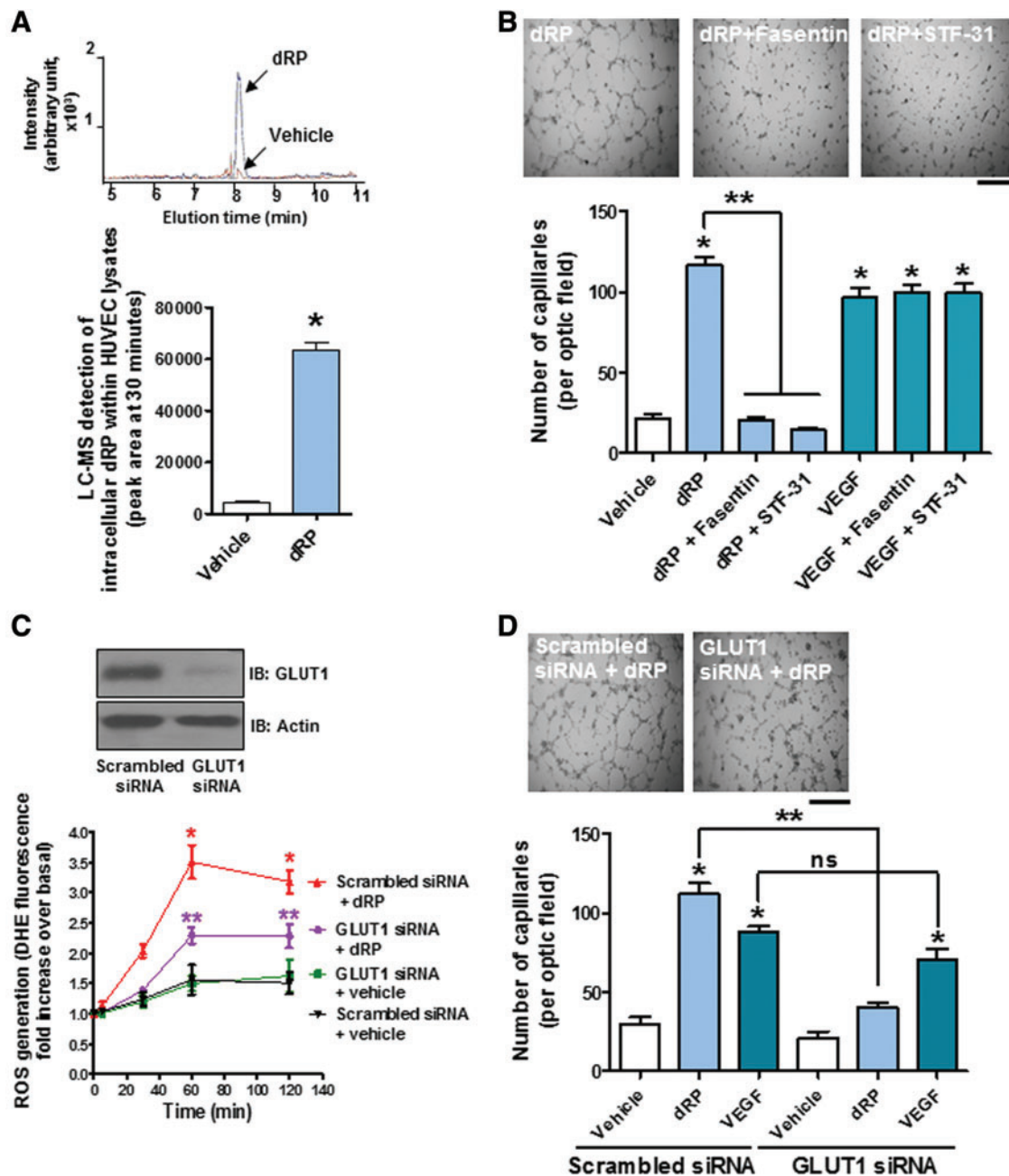


**FIG. 2. dRP stimulates increased levels of ROS generation in HUVECs in an NOX-dependent manner.** Quantitative measurements of superoxide anion production in HUVECs were performed using the cell-permeable superoxide-specific spin probe CMH and EPR. **(A)** Cells were treated for 45 min with 200  $\mu\text{M}$  dRP, vehicle (Tyrode's HEPES buffer), or 50 ng/ml TNF- $\alpha$  in the presence of CMH (200  $\mu\text{M}$ ) before EPR analysis. **(B)** Inhibition of superoxide anion production induced by 200  $\mu\text{M}$  dRP was also detected by EPR by 10  $\mu\text{M}$  MnTBAP, 10  $\mu\text{M}$  Tempol, or 1  $\mu\text{M}$  VAS2870. **(A, B)** Representative EPR traces are shown (i). The bar charts (ii) show superoxide anion production rates ( $\text{pmol mg}^{-1} \text{min}^{-1}$ ) (mean  $\pm$  SEM, one-way ANOVA with Bonferroni *post hoc* test,  $*p < 0.05$ ,  $n = 4$ ). **(C)** Calibration curve obtained using known concentrations of the oxidized spin probe (*i.e.*, CM\*). **(D)** Activation of NOX2 confirmed by coimmunoprecipitation with p47phox. HUVECs were treated with a vehicle or 200  $\mu\text{M}$  dRP for 1 h. NOX2 immunoprecipitates were subjected to immunoblotting for p47phox and NOX2. Blots are representative of four independent experiments. CMH, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; EPR, electron paramagnetic resonance; NOX, NADPH oxidase. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

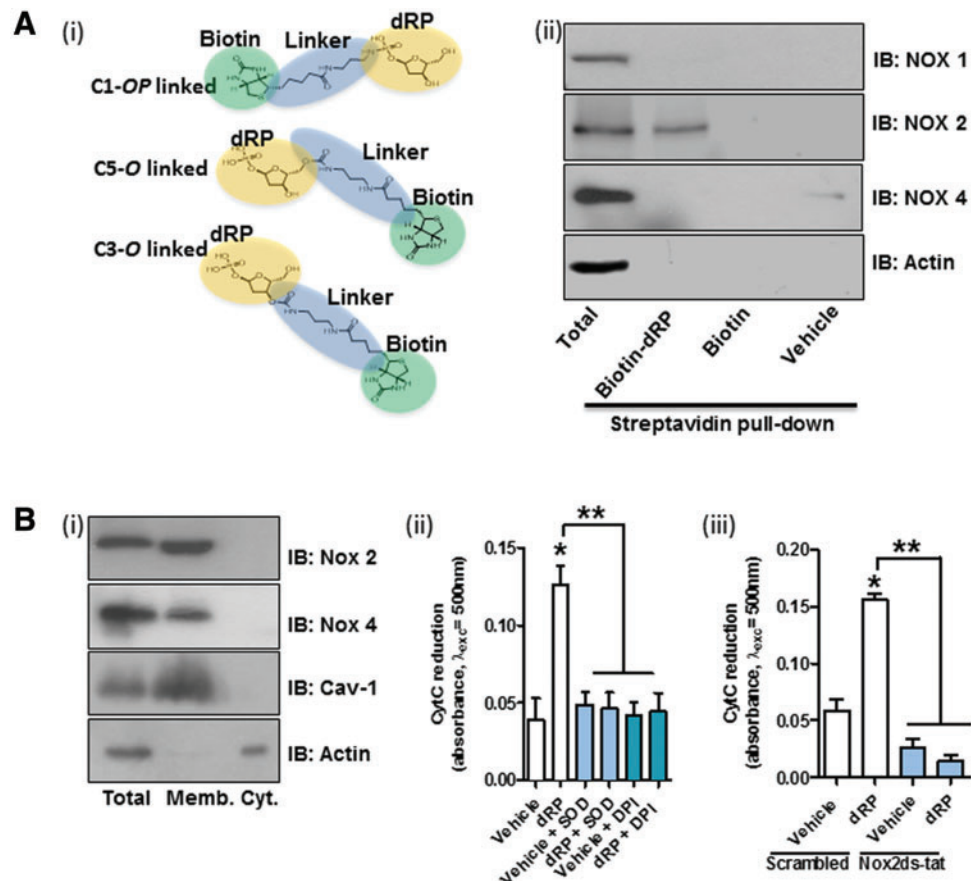




**FIG. 3. dRP stimulates increased levels of ROS generation and tube formation in an NOX-dependent manner.** (A) ROS generation in response to 200  $\mu$ M dRP or vehicle was measured as described above in the presence of 1  $\mu$ M VAS2870. (B) dRP-induced ROS production suppressed by inhibition of NOX2 with 10  $\mu$ M Nox2ds-tat. Immunoblot analysis of NOX2-specific knockdown by siRNA (and scrambled siRNA control). (C) ROS generation in response to 200  $\mu$ M dRP was measured in control (scrambled siRNA) and NOX2 knockdown cells. (D) Representative images of tube formation by cells transfected with scrambled siRNA and NOX2 siRNA in the presence of 200  $\mu$ M dRP are shown. Total number of tubes measured with the Angiogenesis Analyzer plug-in of ImageJ. Representative images (*top*) and data analysis are shown (*bottom*). (E, F) Genetic silencing of NOX4 in HUVECs does not affect dRP-induced ROS generation increase. Tube formation in response to 200  $\mu$ M dRP by scrambled and NOX4 knockdown cells was measured. Time courses (A–C, E) were analyzed by two-way ANOVA ( $n=4$ ) with Bonferroni *post hoc* test [ $*p < 0.05$ , compared with vehicle (A, B) or vehicle/scrambled siRNA (C, E);  $**p < 0.05$  compared with dRP (A, B) or dRP/scrambled siRNA (C, E)]. Bar graphs (D, F) represent quantification of tube number per optical field compared by one-way ANOVA with Bonferroni post-test ( $*p < 0.05$  compared with vehicle,  $n=5$ ). Bar: (D, F) 300  $\mu$ m. siRNA, small interfering RNA. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)



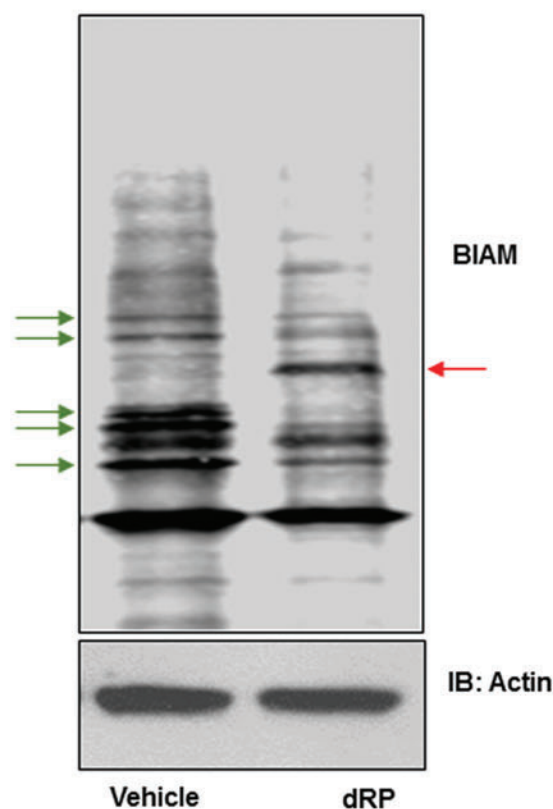
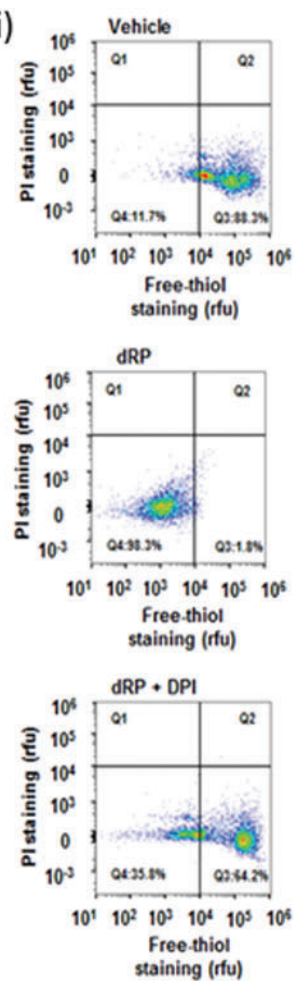
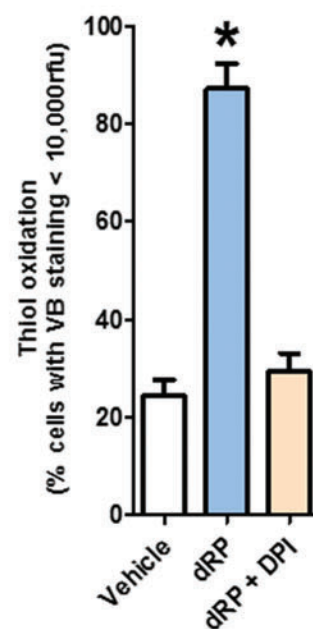
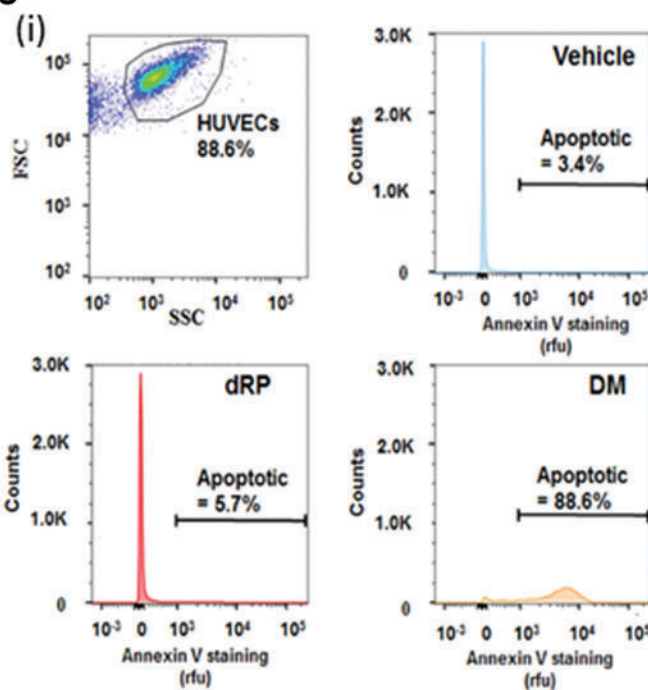
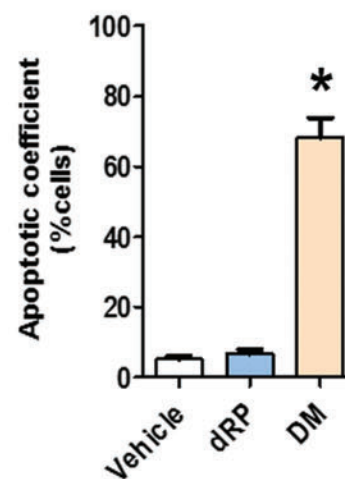
**FIG. 4. dRP acts intracellularly following its internalization by the transporter GLUT1.** (A) LC-MS detection of intracellular dRP. HUVECs were treated with 200  $\mu$ M dRP, before three washes in PBS and ultrasonication. Example chromatogram (upper panel) and data quantification (lower panel) are shown. Statistical significance was tested by *t*-test ( $*p < 0.05$ , compared with vehicle,  $n = 4$ ). (B) STF-31 and fasentin (10  $\mu$ M) inhibited dRP-dependent, but not VEGF-dependent, tube formation. Example pictures (upper panel) and data quantification (lower panel) are shown. Bar graphs represent quantification of tube number per optical field compared by one-way ANOVA with Bonferroni *post hoc* test ( $*p < 0.05$  compared with vehicle,  $n = 5$ ). Bar: 300  $\mu$ m. (C) siRNA-dependent silencing of GLUT1 inhibits dRP-induced ROS generation. Following GLUT1 silencing displayed in top panels, ROS was measured as described over a period of 2 h. Time courses were analyzed by two-way ANOVA ( $n = 4$ ) with Bonferroni *post hoc* test ( $*p < 0.05$ , compared with vehicle/scrambled siRNA;  $**p < 0.05$  compared with dRP/scrambled siRNA). (D) siRNA-dependent silencing of GLUT1 inhibits dRP-induced tube formation. Example pictures for dRP response by HUVECs treated with scrambled siRNA or GLUT1 siRNA are shown in top panels. Bar graphs represent quantification of tube number per optical field compared by one-way ANOVA with Bonferroni *post hoc* test ( $*p < 0.05$  compared with scrambled siRNA/vehicle,  $**p < 0.05$  compared with scrambled siRNA/dRP, ns, nonsignificant,  $n = 4$ ). Bar: 300  $\mu$ m. GLUT1, glucose transporter 1; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

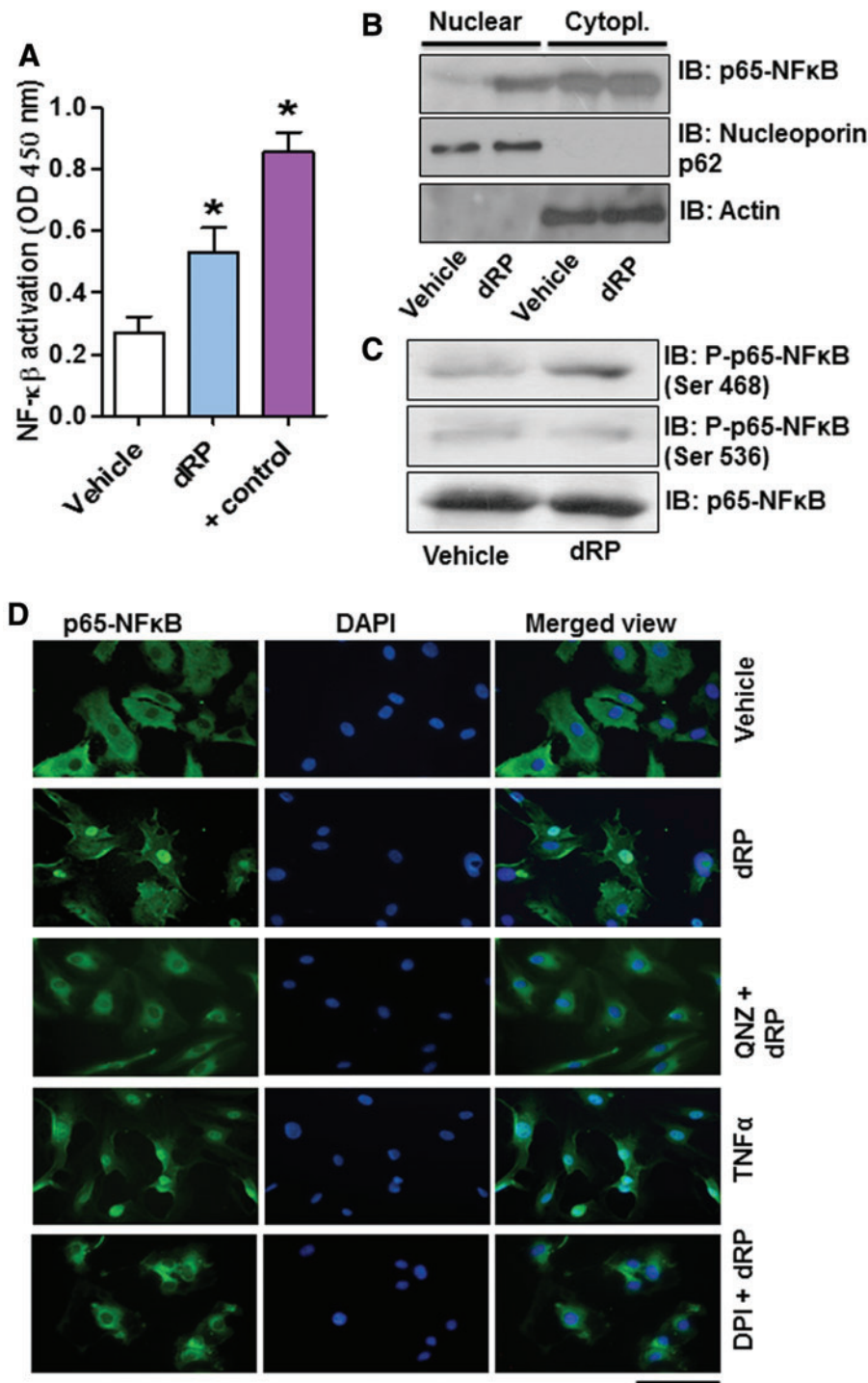


**FIG. 5. dRP directly activates NOX2.** (A) Binding of NOX2 was assessed by pull-down using a mixture of three dRP-biotin conjugates (i). NOX 1, 2, 4, and actin were detected by immunoblotting (ii) (four independent experiments). (B) Membrane fractions treated with dRP display NOX activation. NOX-1, 2, 4, caveolin-1, and  $\beta$ -actin were tested by immunoblotting (i). The membrane fractions were treated with 200  $\mu M$  dRP using cytochrome c reduction assay. Cytochrome c reduction was measured as absorbance at wavelength 550 nm. One thousand units per milliliter SOD was utilized to determine the superoxide anion-dependent component, while DPI (100  $\mu M$ ) was used to determine the role of NOXs and other flavoenzymes (ii). Ten micromolars of Nox2ds-tat (or scrambled peptide as a negative control) was utilized to assess the role of NOX2 in the oxidative response measured by this membrane assay (iii). Data in B were analyzed by one-way ANOVA with Bonferroni post-test [ $*p < 0.05$ , compared with vehicle (ii) or scrambled siRNA/vehicle (iii),  $**p < 0.05$  compared with dRP (ii) or scrambled siRNA/dRP (iii),  $n = 6$ ]. DPI, diphenylene iodonium; SOD, superoxide dismutase. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

**FIG. 6. dRP induces oxidative stress without significantly increasing apoptosis.** (A) HUVECs treated with or without 200  $\mu M$  dRP for 4 h were labeled for 2 h with 20  $\mu M$  BIAM in anoxic conditions. Thiol oxidation status was determined by protein separation using SDS-PAGE and staining with HRP-streptavidin. Green arrows indicate thiol oxidation, while red arrows represent thiol reduction.  $\beta$ -Actin immunoblotting was used to confirm equal loading. Blots are representative of four independent experiments. (B) VitaBright-43<sup>TM</sup> staining was also utilized to measure the level of intracellular reduced thiols. HUVECs were treated with vehicle solution (Tyrode's HEPES buffer) or stimuli with/without NOX inhibitor (200  $\mu M$  dRP, 10  $\mu M$  DPI) for 30 min. Cells were costained with VitaBright-43 and propidium iodide and analyzed by image cytometry using the NucleoCounter NC-3000<sup>®</sup> system. Plots comparing VitaBright-43 (VB) intensity versus propidium iodide intensity are shown (i). Intracellular thiol oxidation was quantified by counting the percent of cells with VitaBright-43 staining below 10,000 rfu (ii). Statistical analysis was performed by one-way ANOVA with Bonferroni post-test ( $*p < 0.05$ , compared with vehicle,  $n = 6$ ). (B) Cell apoptosis was measured by flow cytometry for PE-Annexin V staining. HUVECs were treated with vehicle (Tyrode's HEPES buffer), 200  $\mu M$  dRP, or 5 mM diethyl maleate (positive control) for 12 h. FSC/SSC and Annexin V staining histograms from four independent experiments are presented (i). Data analysis is also shown (ii). Data are mean  $\pm$  SEM, analyzed by one-way ANOVA with Bonferroni post-test ( $*p < 0.05$ , compared with vehicle,  $n = 6$ ). BIAM, biotinyl-iodoacetamide; FSC, forward scattering; HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, side scattering. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)



**A****B (i)****(ii)****C****(ii)**

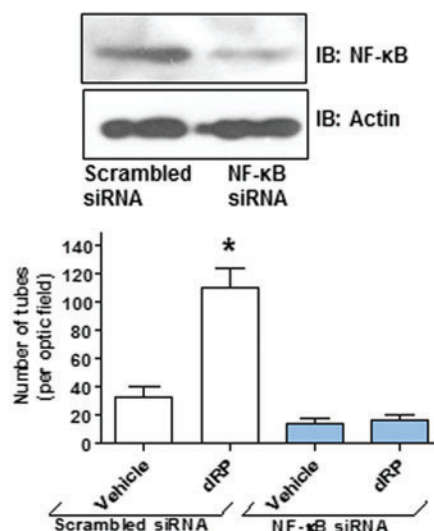
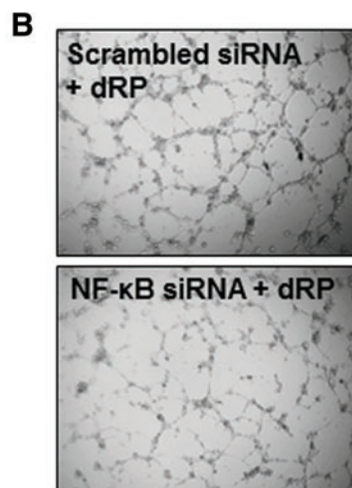
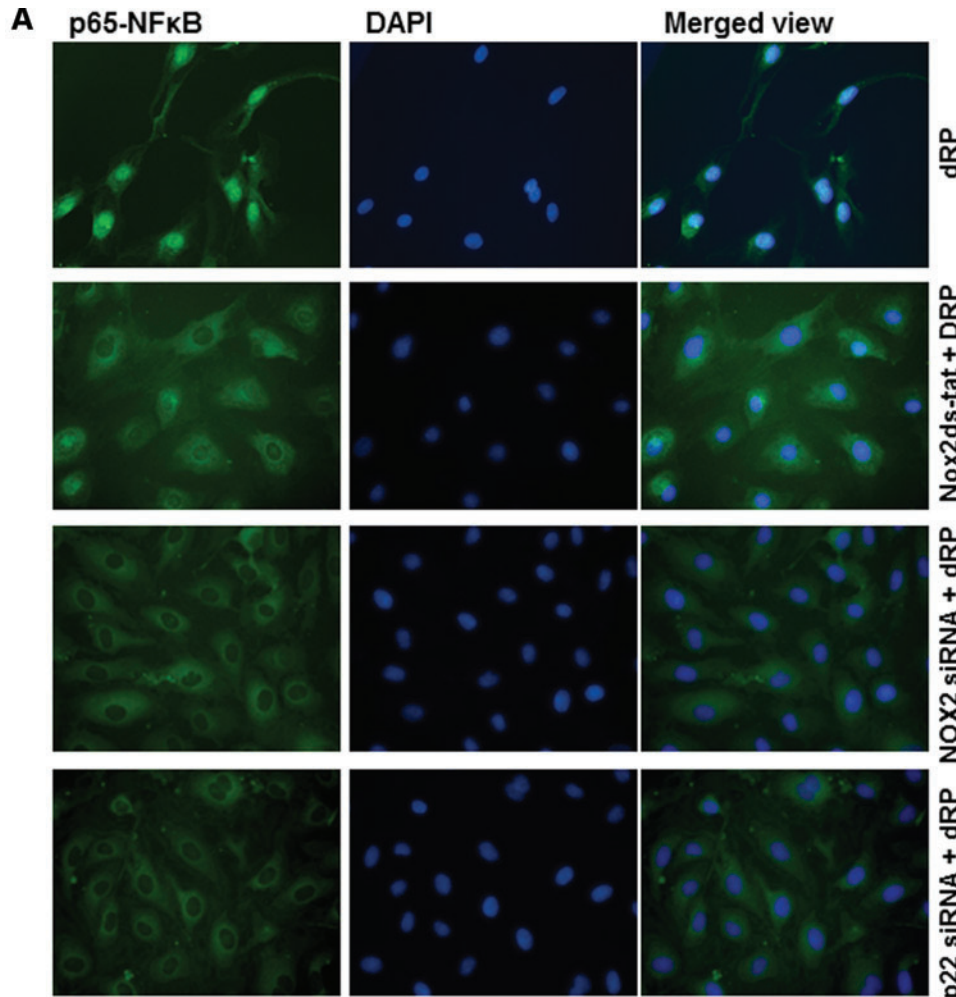


**FIG. 7. NF- $\kappa$ B is activated in response to dRP.** (A) DNA binding capacity of NF- $\kappa$ B (p65 subunit) was determined using TransAM<sup>®</sup> for NF- $\kappa$ B (cat. No. 43296; Active Motif). HUVECs were treated with or without 200  $\mu$ M dRP for 30 min. Whole cell extract from Jurkat cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate and calcium was used as positive control. One-way ANOVA with Bonferroni post-test was used to test statistical significance (mean  $\pm$  SEM,  $n = 6$ ,  $*p < 0.05$ ). (B) The translocation of p65-NF- $\kappa$ B to the nucleus was tested by subcellular fractionation using the NE-PER kit (Pierce). Cytoplasmic and nuclear fractions were immunoblotted for p65-NF- $\kappa$ B, nucleoporin-p62 (a nuclear marker), and  $\beta$ -actin (a cytoplasmic marker). Data are representative of four independent experiments. (C) NF- $\kappa$ B activation was also investigated by phospho-specific immunoblotting. Total cell lysate was immunoblotted with phospho-specific NF- $\kappa$ B antibodies (Ser468 or Ser536) and total NF- $\kappa$ B antibody (as loading control). Immunoblots represent 4 independent experiments. (D) Immunolocalization of p65-NF- $\kappa$ B (green) in dRP-stimulated HUVECs (200  $\mu$ M, 1 h) was also tested. Where indicated, 100 nM QNZ, an inhibitor of NF- $\kappa$ B activation, was added. One hundred micromolars of DPI was used as the NOX inhibitor, whereas 50 ng/ml TNF- $\alpha$  was utilized as a positive control. Images are representative of five independent experiments. Bar: (D) 100  $\mu$ m. NF- $\kappa$ B, nuclear factor kappa B; QNZ, N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

pro-oxidative cell conditions, leading to oxidation of cysteines in several proteins (as expected from the activation of a highly expressed pro-oxidative enzyme such as NOX2). Protein thiol oxidation (Fig. 6B) without induction of cell apoptosis (Fig. 6C) was confirmed by free thiol staining and Annexin V binding, respectively.

#### *dRP induces activation of NF- $\kappa$ B in an NOX-dependent manner*

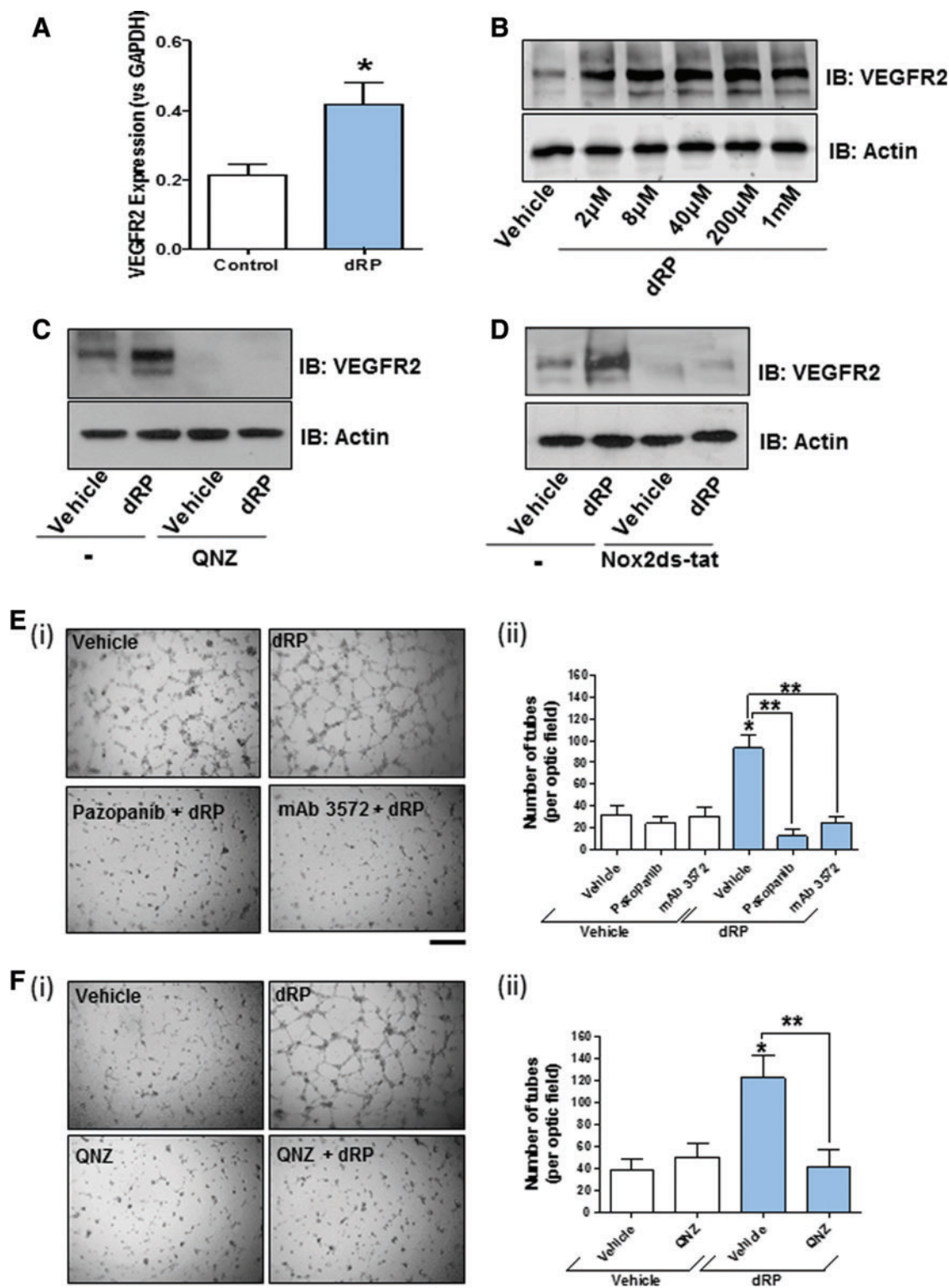
Several redox-dependent transcription factors, including HIF-1 (51), Nrf2 (30, 34), and NF- $\kappa$ B (11, 56), play an important role in endothelial cell responses. To probe the



**FIG. 8. dRP induces NF- $\kappa$ B activation in NOX-dependent manner and NF- $\kappa$ B activity is critical for endothelial tube formation in response to dRP.**

(A) Representative immunofluorescence images of HUVECs stained for NF- $\kappa$ B (green) and DAPI (blue). Where indicated, HUVECs were pretreated with 10  $\mu$ M Nox2ds-tat peptide for 1 h or subjected to NOX2 and p22phox siRNA-mediated genetic silencing for 72 h before treatment with 200  $\mu$ M dRP for 30 min. Images are representative of four independent experiments. (B) Tube formation was assessed by seeding scrambled and NF- $\kappa$ B siRNA-treated cells onto growth factor-reduced Matrigel with or without 200  $\mu$ M dRP. Representative images show tube formation after 4 h (left) and p65-NF- $\kappa$ B downregulation (right). Bar graphs (bottom right) represent quantification of tube number per optical field using ImageJ software with Angiogenesis Analyzer plugin (\* $p$  < 0.05, one-way ANOVA with Bonferroni post-test,  $n$  = 5). Bar: (A) 100  $\mu$ m; (B) 300  $\mu$ m. DAPI, 4',6-diamidino-2-phenylindole. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)





**FIG. 9. VEGFR2 is upregulated in response to dRP in an NF- $\kappa$ B-dependent manner and its activity is necessary for dRP-dependent angiogenesis.** (A) qPCR analysis of VEGFR2 expression on HUVECs treated with or without 200  $\mu$ M dRP for 4 h. The  $2^{-\Delta\Delta C_t}$  analysis method was used to analyze the data with GAPDH used as normalizer. Statistical significance of the difference was tested using a nonparametric Mann-Whitney test (mean  $\pm$  SEM,  $n=4$ ,  $*p<0.05$ ). (B) HUVECs were treated with increasing concentration of dRP (2  $\mu$ M to 1 mM) for 6 h. Alternatively, HUVECs were incubated with (C) 100 nM QNZ or (D) 10  $\mu$ M Nox2ds-tat for 1 h and then stimulated with 200  $\mu$ M dRP for 6 h. Cell lysates were immunoblotted for VEGFR2 and  $\beta$ -actin. Data are representative of four independent experiments. (E) Effects of VEGFR2 inhibitors pazopanib and mAb3572 on dRP-induced tube formation. HUVECs with or without 200  $\mu$ M dRP were tested in the presence of 10  $\mu$ g/ml pazopanib and 50 ng/ml mAb3572 antibody. Representative pictures (i) and quantification (ii). (F) Effects of the NF- $\kappa$ B inhibitor QNZ (100 nM) on dRP-induced tube formation. Representative pictures (i) and quantification (ii). Bar graphs represent quantification of tube number per optical field performed using ImageJ software with Angiogenesis Analyzer plug-in and compared by one-way ANOVA with Bonferroni post-test ( $*p<0.05$ ,  $**p<0.01$ ,  $n=6$ ). Bar: (E, F) 300  $\mu$ m. qPCR, real-time quantitative polymerase chain reaction; VEGFR2, VEGF receptor 2. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)



involvement of HIF-1, Nrf2, and NF- $\kappa$ B as mediators of the proangiogenic activities of dRP, we tested the DNA binding activity of these transcription factors in HUVECs treated with dRP (53). These experiments revealed that NF- $\kappa$ B is strongly activated in the presence of dRP (Fig. 7A), whereas HIF-1 and Nrf-2 did not show any significant activation (Supplementary Fig. S6B). In agreement with these results, dRP increased p65-NF- $\kappa$ B levels in the nuclear fraction of HUVECs (as a result of nuclear translocation; Fig. 7B) and promoted NF- $\kappa$ B phosphorylation on Ser468 (but not Ser536; Fig. 7C and Supplementary Fig. S6A). Translocation of NF- $\kappa$ B to the nucleus upon dRP treatment was confirmed by immunolabeling and confocal imaging (Fig. 7D). Treatment with the nonspecific flavoenzyme inhibitor DPI or direct inhibition of NF- $\kappa$ B with the potent inhibitor N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine (QNZ) (61) abolished nuclear translocation of NF- $\kappa$ B. Similar to DPI, NOX2 inhibition by the peptide Nox2ds-tat and genetic silencing of p22phox or NOX2 abolished translocation of NF- $\kappa$ B to the nucleus in dRP-stimulated HUVECs (Fig. 8A). Collectively, these data suggest that dRP-dependent activation of NOX2 is responsible for NF- $\kappa$ B activation. The functional role of NF- $\kappa$ B in the proangiogenic activity of dRP was tested by siRNA-dependent genetic silencing of this transcription factor (Fig. 8B), which resulted in complete inhibition of endothelial tube formation in response to dRP.

#### *dRP-dependent activation of NF- $\kappa$ B upregulates VEGFR2 and induces endothelial tube formation*

An initial analysis of the expression of angiogenic factors in HUVECs by enzyme-linked immunosorbent assay (ELISA) (Supplementary Fig. S7A) and immunoblotting (Supplementary Fig. S7B) did not detect any significant change in response to dRP, suggesting that the effects of dRP do not depend upon autocrine production of these factors. Further studies, however, showed that VEGFR2 is robustly upregulated at the messenger RNA (mRNA) and protein levels in HUVECs following exposure to dRP (Fig. 9A–D). The NF- $\kappa$ B inhibitor QNZ ablated the expression of VEGFR2 in HUVECs and inhibited the upregulation of this receptor by dRP, suggesting a critical role for NF- $\kappa$ B in the expression of VEGFR2 (Fig. 9C). The NOX2 inhibitor Nox2ds-tat

(54) significantly reduced upregulation of VEGFR2 by dRP (Fig. 9D). The functional relevance of VEGFR2 upregulation for the proangiogenic activity of dRP was confirmed in experiments using the VEGFR2 inhibitor pazopanib and the VEGFR2-specific inhibitory antibody MAB3572, both of which abolished tubulogenesis stimulated by dRP (Fig. 9E). Experiments with the NF- $\kappa$ B inhibitor QNZ suggested that this transcription factor is necessary for dRP-dependent tube formation (Fig. 9F), which correlates with the strong effect of NF- $\kappa$ B inhibition on VEGFR2 expression shown in Figure 9B, and the involvement of VEGFR2 in dRP-dependent angiogenesis shown in Figure 9E.

#### *dRP stimulates VEGFR2 upregulation and NOX2-dependent angiogenesis in vivo*

The proangiogenic activity of dRP has been described previously (35, 38, 49, 50). To confirm that the mechanism of action of dRP that we characterized *in vitro* also occurs *in vivo*, we applied dRP to excisional wounds in mice and assessed the levels of tissue vascularization by hematoxylin staining (Fig. 10A) and VEGFR2 expression at the wound site 7 days after application (Fig. 10B). These experiments showed a significant increase in both wound vascularity and expression of VEGFR2 in wounds treated with dRP. The dependence of dRP proangiogenic activity on NOX2 was then tested in NOX2<sup>-/-</sup> mice. Using a Matrigel plug vascularization assay, we demonstrated that the presence of dRP stimulated a significantly higher vascularization of the plug in wild-type animals compared with NOX2<sup>-/-</sup> mice. This was demonstrated by hematoxylin (Fig. 10C) and endothelial-specific CD31 staining (Fig. 10D).

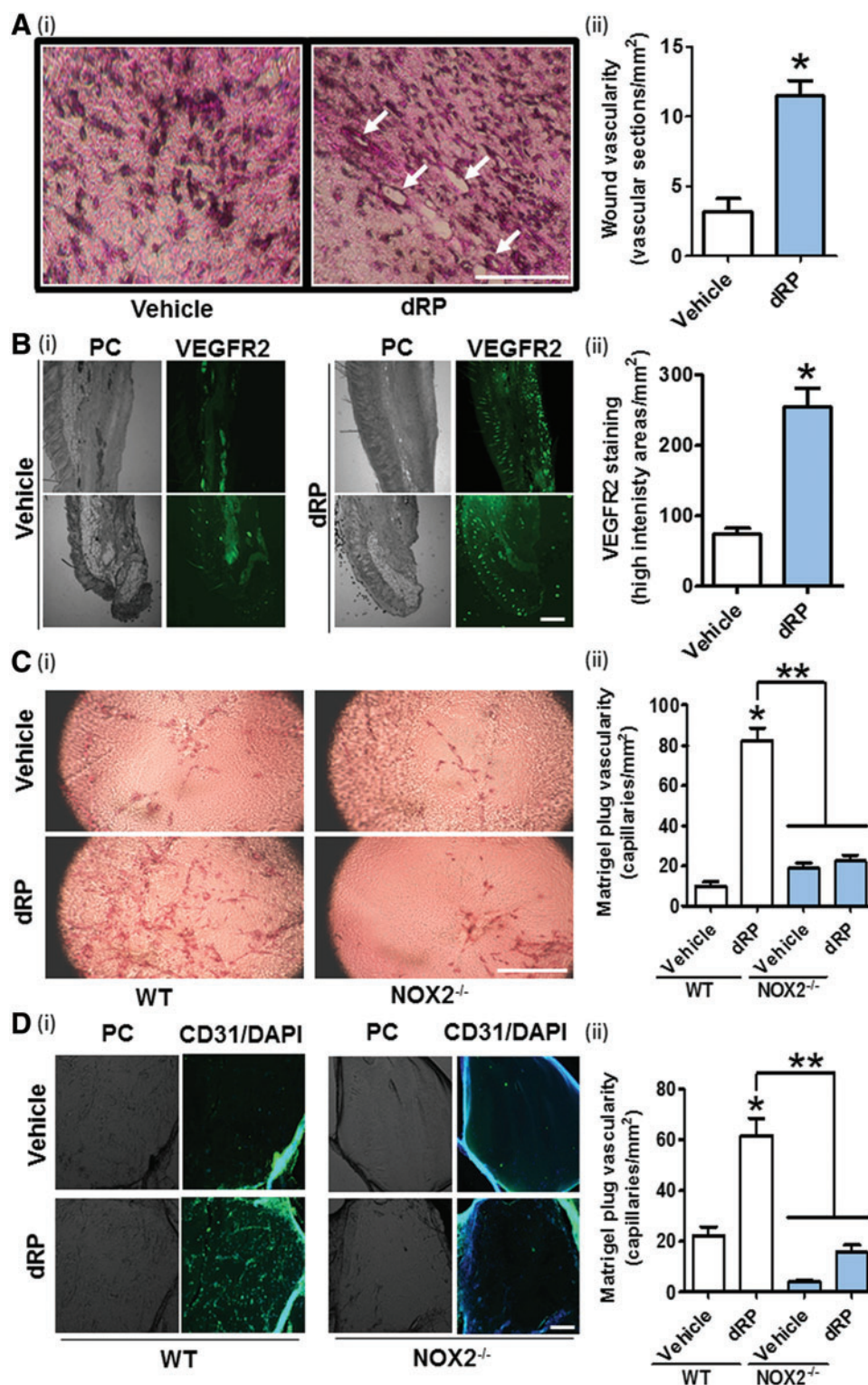
## Discussion

The proangiogenic activity of dRP has been described previously, although the mechanism of action has remained elusive. The dRP-generating enzyme TP was initially cloned as platelet-derived endothelial cell growth factor (PD-ECGF) and characterized for its proangiogenic properties (27). Local injection of TP or TP-expressing cells has been shown to induce tissue neovascularization *in vivo*, which depends on the enzymatic activity of TP and generation of dRP (35). TP

**FIG. 10. dRP stimulates VEGFR2 upregulation and NOX2-dependent angiogenesis *in vivo*.** (A) Representative examples (i) and quantification (ii) of the hematoxylin/eosin staining of wound tissue treated with dRP or vehicle control (PBS). Data represent counts of vascular structures (surrounded by continuous intimal monolayer) per optic field. Statistical significance of the difference was assessed by nonparametric Mann–Whitney test (\* $p$  < 0.05 compared with vehicle,  $n$  = 10). Vascular structures are highlighted by white arrows in the picture. (B) Representative examples (i) and quantification (ii) of the VEGFR2-specific staining of wound tissue treated with dRP or vehicle control (PBS). Data represent counts of high fluorescence intensity areas per mm<sup>2</sup>, as estimated using ImageJ. Statistical significance of the difference was assessed by nonparametric Mann–Whitney test (\* $p$  < 0.05 compared with vehicle,  $n$  = 8). (C) Representative examples (i) and quantification (ii) of hematoxylin staining of Matrigel plugs containing dRP (41.2  $\mu$ g/plug) or vehicle control (PBS) after 7 days of implantation in wild-type (C57BL/6J) or NOX2<sup>-/-</sup> (B6.129S-Cybbtm1Din/J; Jackson Laboratories) mice. Data represent counts of capillary structures per mm<sup>2</sup>, as estimated using ImageJ. Statistical significance of the difference was assessed by one-way ANOVA with Bonferroni *post hoc* test (\* $p$  < 0.05 compared with vehicle, \*\* $p$  < 0.05 compared with dRP,  $n$  = 6). (D) Representative examples (i) and quantification (ii) of the DAPI/CD31-specific staining of Matrigel plugs containing dRP (41.2  $\mu$ g/plug) or vehicle control (PBS) after 7 days of implantation in wild-type (C57BL/6J) or NOX2<sup>-/-</sup> (B6.129S-Cybbtm1Din/J; Jackson Laboratories) mice. Data represent counts of capillary structures per mm<sup>2</sup>, as estimated using ImageJ. Statistical significance of the difference was assessed by one-way ANOVA with Bonferroni *post hoc* test (\* $p$  < 0.05 compared with vehicle, \*\* $p$  < 0.05 compared with dRP,  $n$  = 6). Bars: 300  $\mu$ m (throughout). To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

and other dRP-generating enzymes, such as PNP and UP, are overexpressed in cancers and associated with cancer vascularization and metastasis (31). In our studies, we identified platelets and macrophages as key generators of micromolar concentrations of dRP (49), whereas endothelial cells do not synthesize detectable levels of dRP and recognize this molecule (or its dephosphorylation product 2-deoxy-D-ribose) as a paracrine signal that triggers angiogenesis (6).

In this study, we demonstrate for the first time that the oxidative response induced by dRP in endothelial cells is mediated by NOX2 and that dRP can directly bind and activate this enzyme. Importantly, we showed that dRP binds and pulls down NOX2, but not NOX1 or NOX4, from endothelial cell lysates. As NOX1 and NOX2 complexes share several regulatory subunits (*i.e.*, p22phox, p47phox, and Rac1), the specific pull-down of NOX2, and not NOX1, by biotinylated



dRP supports the hypothesis that dRP directly interacts with the NOX2 enzymatic subunit (also known as gp91phox). Direct binding and activation of the NOX complex are remarkable and rarely reported modes of action for a signaling molecule, which have been described in only a few recent studies. Arachidonic acid has been shown to activate NOX2 by promoting its interaction with the p67phox/Rac1 complex (37). In this case, the authors proposed that arachidonic acid interacts directly with p67phox. The involvement of p47phox, p67phox, or other subunits in the dRP-dependent activation of NOX2 demonstrated in our study cannot be excluded at present, but the initial binding appears to be to NOX2.

A correlation between NOX-dependent ROS generation and angiogenesis has been described previously. NOX4 and NOX2 both play prominent roles in the stimulation of angiogenic responses of endothelial cells (15, 39, 40, 63, 69), whereas NOX1 and NOX5 have received less attention (3, 4). In particular, a link between NOX2 and NOX4 activity and increased endothelial cell motility has been reported (26, 45). In our study, NOX2 activation by dRP leads to NF- $\kappa$ B activation (as shown by several approaches, including p65-NF- $\kappa$ B translocation and phosphorylation), which appears critical in the proangiogenic signaling cascade triggered by dRP. The role of NF- $\kappa$ B in the signaling cascade stimulated by dRP was demonstrated using both the QNZ inhibitor [which not only has low nanomolar potency on NF- $\kappa$ B but can also inhibit store-operated calcium channels at high nanomolar concentrations (68)] and genetic silencing of p65-NF- $\kappa$ B. This is in agreement with recent studies showing activation of NF- $\kappa$ B downstream of NOX2 in angiogenic responses of endothelial cells (40). However, in contrast to this latter study, we did not detect any significant changes in the expression of VEGF-A or angiopoietin-2 in response to dRP treatment. One possible explanation for these contrasting findings is the source of endothelial cells used in the two studies (*i.e.*, human umbilical vein *vs.* human pulmonary microvasculature). Instead, we identified VEGFR2 as a key component of the NF- $\kappa$ B-mediated angiogenic response stimulated by dRP. This is in keeping with previous studies showing that the promoter of VEGFR2 contains NF- $\kappa$ B binding motives (55) and recent studies showing regulation of VEGFR2 expression by NF- $\kappa$ B (17). Importantly, we confirmed VEGFR2 upregulation by dRP *in vivo* using a mouse excisional wound assay.

Several previous studies have highlighted associations between endothelial cell oxidative status and the angiogenic response. For example, the link between ROS formation in hypoxic conditions and the activity of HIF-1 has been described (22). Although additional routes have also been proposed (2), ROS have been shown to promote HIF-1-dependent transcription *via* inactivation of prolyl hydroxylase and reduction of HIF proteasome-dependent degradation (47). The activation of NOX4 and NOX3, but not NOX2, has previously been associated with HIF-1 activation (8, 69), whereas NOX2 has been shown to stimulate NF- $\kappa$ B activation in endothelial cells (40). Since we have identified NOX2 as a key target of dRP biological activity, our data support the existing literature showing that NF- $\kappa$ B, as opposed to HIF-1, is activated by this member of the NOX family. We confirmed that NOX2 is also necessary for the proangiogenic effect of dRP *in vivo*. This is in line with two recent studies showing a role for this member of the NOX family in the

stimulation of angiogenesis *in vivo*, although it should be emphasized that these reports, in contrast to the present study, did not provide clarity on the underlying molecular mechanisms (10, 70).

Interestingly, we observed direct cysteine oxidation in endothelial cells treated with dRP (as demonstrated by the loss of free thiols). The product of cysteine oxidation by ROS in mammalian cells is cysteine sulfenic acid, which can undergo further oxidation to generate cysteine sulfinic acid and cysteine sulfonic acid or can form intramolecular or intermolecular disulfide bridges (16). The oxidation of cysteine by ROS has important functional consequences for protein phosphatases and protein kinases (52). For example, and relevant to our study, ROS-induced serine and/or tyrosine phosphorylation, ubiquitination, and consequent proteolytic degradation of the inhibitor subunit, inhibitor of NF- $\kappa$ B (I $\kappa$ B), are responsible for nuclear transportation and activation of NF- $\kappa$ B (43). These observations support our data showing ROS-dependent activation of NF- $\kappa$ B in response to dRP.

Overall, this study has comprehensively elucidated the mechanism of action of dRP, (Supplementary Fig. S8) a small molecule with proangiogenic effects *in vitro* and *in vivo* (49). We showed that similarly to other sugars (either hexoses or pentoses) (29, 44), dRP is readily internalized by endothelial cells and that the transporter GLUT1 is the most likely transporter responsible for this. This is in agreement with previous studies suggesting that TP stimulates endothelial cells in a nonreceptor-mediated manner (6). Once internalized, dRP has the remarkable ability to directly bind and activate NOX2, but not NOX4 or NOX1. Activation of NOX2 and the resulting generation of ROS trigger NF- $\kappa$ B activation and promote angiogenic responses in endothelial cells, which depend on the activity of VEGFR2. This study, therefore, represents a milestone in the understanding of the proangiogenic activity of the dRP-generating enzyme TP, which was cloned as platelet-derived endothelial growth factor and characterized as a potent stimulator of angiogenesis in the late 1980s (27).

## Materials and Methods

### LC-MS detection and quantification of dRP

Macrophages were differentiated from whole bone marrow cultures from B57BL6 mice in Dulbecco's modified Eagle's medium +10% L929 cell-conditioned medium, 10% fetal bovine serum (FBS), and 1 $\times$  penicillin/streptomycin. Activation of macrophages was performed with 100 ng/ml LPS and 100 ng/ml interferon gamma (IFN $\gamma$ ) or 20 ng/ml interleukin 4 (IL4) and 50 ng/ml IFN $\gamma$ . Platelets were isolated *via* two-step centrifugation, as previously described (66). Activation was obtained with 0.1 U/ml thrombin or 10  $\mu$ g/ml collagen for 10 min in an aggregometer at 700 rpm. Endothelial cells with or without dRP incubation (200  $\mu$ M) were washed with phosphate-buffered saline (PBS) three times and lysed by ultrasonication. For all samples (*i.e.*, cell extracts obtained by ultrasonication or culture supernatants), proteins were eliminated by acetonitrile precipitation. Quadrupole time-of-flight (QTOF)-UHPLC analysis was conducted using a MaXis HD electrospray ionization (ESI)-QTOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), which was coupled to an Ultimate 3000 UHPLC (Thermo Fisher Scientific). LC was performed using a Dionex

Acclaim RSLC PolarAdvantage II (PA2), 2.2  $\mu$ M, 120 $\text{\AA}$ , 2.1  $\times$  50 mm reverse-phase column (Thermo Fisher Scientific) with a flow rate of 0.4 ml/min and an injection volume of 10  $\mu$ l. Mobile phases A and B consisted of 1 mM ammonium fluoride in water and methanol, respectively. dRP was detected as [M-H]<sup>-</sup> ion with a mass-to-charge (m/z) ratio of 314.0120  $\pm$  0.005 Da.

#### *Endothelial cell culture*

HUVECs were isolated from umbilical veins as described previously (25). HUVECs were cultured and passaged in medium M199 supplemented with 20% FBS, 4 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 20 mM NaHCO<sub>3</sub> and cultured in flasks precoated with 1% gelatin (w/v).

#### *In vitro capillary-like tube formation assay*

Growth factor-reduced (GFR) Matrigel was utilized to provide extracellular matrix for cell culture; 10<sup>4</sup> cells/well in 100  $\mu$ l of M199 (no FBS) were added to 96-well microplates containing 65  $\mu$ l GFR Matrigel per well. Phase-contrast images were captured 4 h after treatment using an EVOS FL microscope with a 4x/0.1 Plan-Achromat objective. Total number of tubes was measured using the Angiogenesis Analyzer plug-in of ImageJ.

#### *Intracellular live-cell ROS generation assay*

HUVECs were plated into 96-well, black, optically clear bottom, tissue culture sterile plates and were cultured for 24 h to reach  $\sim$ 70% confluence. Cells were washed once with PBS, pretreated with inhibitors for 30 min (as indicated in Figs. 1D, 3A–C, 3E, and 4C), and then treated with 5  $\mu$ M DHE and dRP at the desired concentrations. Fluorescence was monitored using a CLARIOstar<sup>®</sup> plate reader at 37°C with atmospheric control at 5% CO<sub>2</sub>/20% O<sub>2</sub>. Fluorescence was measured at Ex/Em: 510/610 nm over 2 h.

#### *Superoxide detection using EPR*

HUVECs cultured in six-well plates were washed twice with EPR-Krebs HEPES buffer (EPR-KHB) adjusted to pH 7.4 and then incubated for 1 h in deoxygenated EPR-KHB (+1 g/l glucose) in the presence of treatments. Cell-permeable superoxide-specific spin probe CMH at a final concentration of 200  $\mu$ M (from 10 mM CMH stock solution in the presence of 25  $\mu$ M deferoxamine and 5  $\mu$ M DETC) was then added for 45 min. The supernatant was then analyzed using a Bruker e-scan with the following settings: center field 1.99 g, microwave power 20 mW, modulation amplitude 2 G, sweep time 10 s, number of scans 10, and field sweep 60 G. Calibration curve was calculated using known concentrations of CM• (CM-radical, No. NOX-18.2; purchased from Noxygen), as shown in Figure 2C. Data are presented as pmols of superoxide anion divided by incubation time in minutes and amount of protein per well in mg, calculated using the bicinchoninic assay method.

#### *Determination of NOX activity in endothelial membranes*

The membrane fraction of HUVECs was separated by ultrasonication and ultracentrifugation (100,000 g for

60 min). NOX activity in the membrane fraction was measured as previously described (9). Superoxide production was measured in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) containing 100  $\mu$ g of the membrane fraction, 10 mM diethyldithiocarbamate, 100  $\mu$ M NADPH, 80  $\mu$ M acetylated cytochrome C, 1000 U/ml catalase, and 100  $\mu$ M EDTA; 10  $\mu$ M DPI and 1000 U/ml SOD were used to inhibit and scavenge ROS, respectively.

#### *ELISA-based assay for transcription factor activation*

DNA binding capacity of NF- $\kappa$ B was determined in whole extracts of HUVECs treated as indicated using the TransAM<sup>®</sup> method according to the suppliers' instructions.

#### *Immunofluorescence of NF- $\kappa$ B translocation*

HUVECs were grown on coverslips to 60–70% confluence and treated with stimuli/inhibitors. Following fixation in 4% w/v paraformaldehyde, cells were stained with anti-p65-NF- $\kappa$ B (1:50), Alexa Fluor<sup>®</sup> 488 rabbit anti-mouse (1:200), and 4',6-diamidino-2-phenylindole (DAPI, 1:100). Slides were examined using the 60 $\times$ 1.40 NA oil objective on an LSM 510 META confocal microscope (Carl Zeiss AG, Jena, Germany).

#### *Real-time quantitative polymerase chain reaction*

Real-time quantitative polymerase chain reaction (qPCR) was performed on a ViiA7 Real-Time PCR System using Power SYBR Green PCR and 300 nM gene-specific primers (VEGFR2: 5'-CCAGTGTCATTTCCGATCACT TT and 5'-GGCCCAATAATCAGAGTGGCA and GAPDH: 5'-AGC CGCATCTTCTTT TGCCT and 5'-TGACGAACATGGG GGCATCA). The amplification of a single PCR product was confirmed by melting curve analysis. Gene-specific mRNA levels were estimated by the 2<sup>− $\Delta\Delta$ Ct</sup> analysis and normalized against GAPDH levels to obtain relative changes in gene expression.

#### *Immunoprecipitation and immunoblotting*

For immunoprecipitation, HUVECs cultured in six-well microplates were lysed in 500  $\mu$ l of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100 containing protease and phosphatase inhibitors). The suspension was ultrasonicated using a 150VT Ultrasonic Homogenizer (BioLogics, Manassas, VA). Primary antibody and protein A/G Plus Agarose were used for immunoprecipitation. Immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride for immunoblotting. For immunoblotting, cells were lysed in RIPA buffer (1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4).

#### *siRNA-mediated gene silencing*

HUVECs were transfected with NOX2 (sc-35503), NOX4 (sc-41586), p65 NF- $\kappa$ B (sc-29410), p22phox (sc-36149), or scrambled control (sc-37007) siRNAs (Santa Cruz Biotechnology). The siRNAs were diluted to 100 nM in transfection medium (sc-36868; Santa Cruz Biotechnology)



containing transfection reagent (sc-29528; Santa Cruz Biotechnology), incubated for 45 min at room temperature, and then further diluted to 20 nM in transfection medium. The cells were covered with this solution and incubated for 3 h at 37°C. The solution was then replaced with fresh culture medium and cells used for experiments 72 h after transfection.

#### *Preparation of a biotinylated dRP bait and streptavidin pull-down assay*

dRP at 0.6 mg/ml was resuspended in anhydrous dimethyl sulfoxide (DMSO) and 1,1'-carbonyldiimidazole. The resulting solution was stirred for 24 h. In a separate vessel, N-(+)-biotinyl-3-aminopropylammonium trifluoroacetate was taken up in anhydrous dimethylformamide and N,N-diisopropylethylamine added. After 30 min, the solvents were evaporated to dryness and the DMSO solution of activated dRP was added. The resulting solution was stirred for 72 h before being divided into 10 equal portions and the solvents removed under vacuum. HUVECs were scraped on ice into 10 mM Tris plus 0.3 M sucrose buffer (pH 7.1). The suspension was sonicated on ice and centrifuged at 1000 g for 5 min. The lysate was treated with biotinylated-dRP or biotin as control for 1 h under rotation at 4°C. Streptavidin beads were added to samples, which were further rotated for 2 h at 4°C. Samples were boiled, loaded on to SDS-PAGE gels, and resulting blots probed for NOX 1, 2, and 4 and for  $\beta$ -actin as loading control.

#### *Intracellular free thiol monitoring by cysteine labeling*

BIAM was used to label free thiols in cell lysates as previously described (12). Cell lysates were obtained by sonication in anoxic conditions (cell lysis buffer: 150 mM NaCl, 0.5% v/v Triton-X, 50 mM Tris-HCl, pH 6.5, Complete™ protease inhibitors). Cell lysates were labeled for 2 h with 20  $\mu$ M BIAM in anoxic conditions. BIAM was then quenched with 10 mM  $\beta$ -mercaptoethanol. Cell proteins were separated by SDS-PAGE, stained with streptavidin-HRP, and visualized by enhanced chemiluminescence.

Alternatively, VitaBright-43™ was used to label free thiol groups on proteins (59). This reagent is cell permeable and gives thioester-coupled fluorescent products in a quantitative manner, which allows an estimate of cell oxidative state;  $5 \times 10^6$  cells/ml treated as described were labeled with VitaBright-43 and propidium iodide, as suggested by suppliers. NucleoCounter3000 (Chemometec A/S) was utilized to analyze cell fluorescence levels associated with the two dyes.

#### *Flow cytometry analysis of apoptosis*

Staining with Annexin V-fluorescein isothiocyanate (FITC) to detect cell surface exposure of phosphatidylserine was performed to examine apoptosis in cultured HUVECs treated with or without dRP for 24 h (50). During apoptosis, an early and ubiquitous event is the exposure of phosphatidylserine at the cell surface, which is detected with Annexin V-FITC. Cells treated with 5 mM diethyl maleate for 24 h served as a proapoptotic control. After incubation, cells were harvested with the gentle dissociating buffer TrypLE®, pelleted by centrifugation, washed with PBS, and resuspended in

PBS. Cell suspensions were stained with Annexin V/FITC according to the manufacturer's instructions. Briefly, PBS-washed cells were suspended in 100  $\mu$ l FITC binding buffer at a minimum concentration of  $1 \times 10^6$  combined with 5  $\mu$ l Annexin V/FITC. After 15 min of incubation in the dark on ice, cells were centrifuged at 2000 rpm for 10 min, resuspended in PBS, and analyzed using FACSCanto II (BD Biosciences). Cells that were Annexin V negative were considered viable cells. Cells positive for Annexin V were considered apoptotic. All samples were prepared in triplicate.

#### *Mouse wound healing*

Mouse maintenance and experimental procedures were performed according to local ethics approval and a dedicated UK Home Office Project license. Wounding was performed as described by Mahdipour and Mace (36). dRP pellets were produced by diluting dRP into PBS:methyl cellulose (1%; 1:1). Fifty microliters of this solution was then spotted and dried to form a pellet containing 10.3  $\mu$ g (25 nmol) of dRP. Control pellets were generated in the same way by adding vehicle solution (PBS) instead of dRP. Pellets were administered immediately following wounding and rehydrated directly into the wound. Pellets were subsequently administered every other day on days 2, 4, and 6. Wounds were harvested at day 7, as appropriate, from sacrificed animals with a 2-mm border, fixed in formalin, and embedded in paraffin. Tissues were sectioned using a Leica CM3050 S to produce serial sections of 10- $\mu$ m thickness. Following dewaxing in xylene/EtOH/MetOH, sections were stained with anti-VEGFR2/KDR/Flk-1 antibodies (1:100, No. AF644; R&D Systems) and FITC-labeled anti-goat IgG secondary antibodies (1:200; Life Technologies). Images were captured using an EVOS FL microscope with a 4x/0.1 Plan-Achromat objective (red fluorescence channel and phase contrast). Alternatively, tissue sections were cut at 5- $\mu$ m thickness and stained with hematoxylin/eosin using Mayer's method (20). Sections were evaluated for vascularity by imaging using an Olympus CKX41 microscope with UPlanFl 4x/0.13 objective.

#### *Matrigel plug vascularization assay*

Mouse maintenance and experimental procedures were performed according to local ethics approval and a dedicated UK Home Office Project license. Wild-type (C57BL6/J) and NOX2<sup>-/-</sup> (B6.129S-Cybbtm1Din/J; Jackson Laboratories) mice were injected with 200  $\mu$ l of growth factor-reduced Matrigel (Corning) containing 41.2  $\mu$ g (100 nmol) of dRP or an equivalent volume of vehicle solution (PBS). Seven days after injection, the animals were euthanized and the Matrigel plug was explanted, fixed in 10% formalin, and embedded in paraffin. Tissues were sectioned using a Leica CM3050 S to produce serial sections of 50- $\mu$ m thickness. Following dewaxing in xylene/EtOH/MetOH, sections were stained either with hematoxylin or anti-CD31 antibodies (1:100, No. 3528; Cell Signaling Technologies), TRITC-labeled anti-mouse IgG secondary antibodies (1:200; Life Technologies), and 0.25  $\mu$ g/ml DAPI. Images were captured using an EVOS FL microscope with a 4x/0.1 Plan-Achromat objective (red and blue fluorescence channels and phase contrast). Alternatively, tissue sections were cut at 5- $\mu$ m thickness and stained

with hematoxylin using Mayer's method (20). Sections were evaluated for vascularity by imaging using an Olympus CKX41 microscope with UPlanFI 4x/0.13 objective.

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### Authors' Contributions

D.V. and G.P. designed, performed, and analyzed experiments. J.M.W. performed chemistry experiments to conjugate dRP to agarose beads. T.M.F. performed qPCR experiments. H.M. provided expertise and logistics for HUVEC isolation. M.B. and K.M. performed experiments for the quantification of dRP in macrophages. S.R. and A.L. performed LC-MS for dRP quantification. C.P.D.W.-J. was involved in the initial conception of the study, provided expertise for HUVEC isolation and assessment of angiogenesis *in vitro*, and commented on the manuscript. G.P. wrote the manuscript.

### Author Disclosure Statement

No competing financial interests exist.

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#### Abbreviations Used

BIAM	= biotinyI-iodoacetamide
CMH	= 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
DAPI	= 4',6-diamidino-2-phenylindole
DHE	= dihydroethidium
DMSO	= dimethyl sulfoxide
DPI	= diphenylene iodonium
dRP	= deoxyribose-1-phosphate
ELISA	= enzyme-linked immunosorbent assay
EPR	= electron paramagnetic resonance
FBS	= fetal bovine serum
FITC	= fluorescein isothiocyanate
GFR	= growth factor-reduced
GLUT1	= glucose transporter 1
HIF-1	= hypoxia-induced factor 1
HRP	= horseradish peroxidase
HUVECs	= human umbilical vein endothelial cells
IFN $\gamma$	= interferon gamma
KHB	= Krebs HEPES buffer



**Abbreviations Used (Cont.)**

LC-MS = liquid chromatography–mass spectrometry  
LPS = lipopolysaccharide  
MnTBAP = Mn(III)tetrakis(4-benzoic acid)porphyrin  
mRNA = messenger RNA  
NAC = N-acetyl-L-cysteine  
NF- $\kappa$ B = nuclear factor kappa B  
NOX = NADPH oxidase  
Nrf-2 = nuclear factor erythroid 2-related factor 2  
PBS = phosphate-buffered saline  
PNP = purine nucleoside phosphorylase  
QNZ = N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine

qPCR = real-time quantitative polymerase chain reaction  
QTOF = quadrupole time-of-flight  
ROS = reactive oxygen species  
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis  
siRNA = small interfering RNA  
SOD = superoxide dismutase  
Tempol = 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl  
TP = thymidine phosphorylase  
UP = uridine phosphorylase  
VEGF = vascular endothelial growth factor  
VEGFR2 = VEGF receptor 2