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Title Page

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Nitro-oleic acid regulates endothelin signaling in human endothelial cells

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Running Title Page

Running title: OA-NO_2 induces ET-B via Nrf2

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Abbreviations used: ARE, Antioxidant Response Element; HUVEC, Human umbilical vein endothelial cells; ChIP, Chromatin immunoprecipitation, ET-1, *EDN1*, Endothelin-1; ET-A, *EDNRA,* Endothelin receptor A; ET-B, *EDNRB*, Endothelin receptor B; GCLM, Glutamate-cysteine ligase modifier subunit; HAEC, Human aortic endothelial cells; HASMC, Human aortic smooth muscle cells; HMOX1, Heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor-E2-related factor 2; OA-NO_2, Nitro-oleic acid; PAH, pulmonary arterial hypertension; siRNA, Small interfering RNA

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Abstract

Nitro-fatty acids are reactive signaling mediators that are formed when unsaturated fatty acids react with nitric oxide or nitric oxide-derived species. Nitro-fatty acids can modify specific signaling pathways via post-translational modifications of cysteine residues in key regulatory proteins. One of the signaling cascades activated by nitro-fatty acids is the Keap1-Nrf2 pathway. We have previously studied the effects of nitro-oleic acid (OA-NO_2) on the human endothelial cell transcriptome. We observed that endothelin receptor B (ET-B, *EDNRB*), the receptor mediating the vasodilatory effects of endothelin-1 (ET-1) is induced by OA-NO_2. Inasmuch as ET-1 is one of the key regulators of vascular tone, we chose to examine in more detail the effect of OA-NO_2 on endothelin signaling in human endothelial cells. Nrf2 was found to regulate the OA-NO_2 induced transcription of ET-B in human and mouse endothelial cells. Furthermore, ChIP analysis revealed that OA-NO_2 increased binding of Nrf2 to an Antioxidant Response Element in the enhancer region of *EDNRB* gene. In addition, we show that both OA-NO_2 and Nrf2 overexpression substantially decreased, and Nrf2 silencing increased the ET-1 concentration in the culture media of endothelial cells. The change in the extracellular ET-1 concentration was dependent on ET-B receptor expression. These data suggest that OA-NO_2 modulates endothelin signaling by increasing Nrf2-dependent expression of the ET-B receptor in endothelial cells, which in turn mediates the decrease in extracellular ET-1 concentration. Based on these results, we propose that OA-NO_2 and Nrf2 may alleviate vasoconstrictive effects of ET-1 by removing it from the circulation.

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Introduction

Nitro-fatty acids are endogenous signaling molecules formed *in vivo* when unsaturated fatty acids react with nitric oxide and nitric oxide derived species (Schopfer *et al.*, 2011). Nitro-fatty acids are generated in inflammatory conditions including ischemic preconditioning (Nadtochiy *et al.*, 2009) and myocardial ischemia/reperfusion (V Rudolph *et al.*, 2010). Nitro-fatty acids can alter specific signaling pathways by Michael addition with nucleophiles of biological targets. They can modulate regulatory protein functions via post-translational modification of susceptible nucleophilic amino acids, such as cysteines (Cys) (Batthyany *et al.*, 2006; Baker *et al.*, 2007; Schopfer *et al.*, 2010; Kansanen *et al.*, 2011). Nitro-oleic acid (OA-NO_2) is beneficial in murine models of vascular disease (Cole *et al.*, 2009; TK Rudolph *et al.*, 2010), type 2 diabetes (Schopfer *et al.*, 2010), and both myocardial (V Rudolph *et al.*, 2010) and renal (Wang *et al.*, 2010) ischemia reperfusion injury. In addition, OA-NO_2 has antihypertensive effects in AngII induced mouse hypertension (Zhang *et al.*, 2010), and it also has antihypertensive signaling actions via inhibition of the enzymatic activity of epoxyeicosatrienoic acid (EET) hydrolyzing soluble epoxide hydrolase by adduction to Cys521 in the vicinity of its catalytic center (Charles *et al.*, 2014).

Nuclear factor-E2-related factor 2 (Nrf2) is a transcription factor that regulates a multiple antioxidant and cytoprotective genes. The well-known Nrf2 target genes that are often used as markers for Nrf2 activation are heme oxygenase-1 (*HMOX1*), glutamate-cysteine ligase *(GCL)* and NAD(P)H quinone oxidoreductase-1 (Kwak *et al.*, 2003; Lee *et al.*, 2003). Kelch-like ECH-associated protein 1 (Keap1) is a redox-regulated protein that inhibits the nuclear translocation of Nrf2 by mediating the rapid ubiquitination and degradation of Nrf2 in non-stimulated, basal conditions (Zhang and Hannink, 2003). In oxidative or electrophilic stress, specific Cys residues in Keap1 are modified, which results in conformational change in Keap1 leading to the escape of Nrf2 from the Keap1-dependent degradation pathway and translocation to the nucleus. In the nucleus, Nrf2 binds to the Antioxidant Response Element (ARE) located in the

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enhancer region of its target genes thus driving their expression (Kansanen *et al.*, 2012). We have previously discovered that OA-NO_2 induces Nrf2-dependent cytoprotective gene expression (Kansanen *et al.*, 2009), which involves direct modification of Keap1 Cys residues Cys38, Cys226, Cys257, Cys273, Cys288, and Cys489. Of these Keap1 cysteine residues, Cys273, Cys288 were found to be functionally most important in the activation of Nrf2 (Kansanen *et al.*, 2011). Furthermore, in a genome-wide analysis of Nrf2-dependent and independent effects of OA-NO_2, we found that the expression of endothelin receptor B (ET-B, gene name *EDNRB*) was induced by OA-NO_2 and repressed by Nrf2 siRNA in human endothelial cells (Kansanen *et al.*, 2009). ET-B is a receptor for endothelin-1 (ET-1, gene name *EDN1*), which was first identified as a potent vasoconstrictor, but it is now recognized that ET-1 can also function as a vasodilator depending on the receptor being activated. In the vasculature, endothelin receptor A (ET-A, gene name *EDNRA*) is present predominantly in smooth muscle cells, whereas the ET-B receptor is located in endothelial cells. However, a sub-family of ET-B receptors is also present in vascular smooth muscle cells. In smooth muscle cells, activation of both ET-A and ET-B induces vasoconstriction, but the stimulation of ET-B receptors in endothelial cells promote vasodilatation (Schneider *et al.*, 2007). In addition, ET-B functions as a clearance receptor to remove ET-1 from the circulation (Kelland, Bagnall, *et al.*, 2010).

Inasmuch as ET-1 is one of the key regulators of vascular tone, we chose to examine in more detail the effect of OA-NO_2 on endothelin signaling in human endothelial cells. We show that the upregulation of ET-B receptor by OA-NO_2 is tightly regulated by Nrf2 in human and mouse endothelial cells. In addition, *in silico* screening identified two putative ARE sites residing on the active enhancer region at the *EDNRB* gene locus. In response to OA-NO_2, ChIP analysis revealed an increase in binding of Nrf2 to an ARE site located 5253 base pairs upstream of transcription start site of *EDNRB* gene. In addition, we show that both OA-NO_2 and Nrf2 overexpression substantially decreased, and Nrf2 silencing increased the ET-1 concentration in the cell culture media. The decrease in the extracellular ET-1 concentration was dependent

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on ET-B receptor expression. These data suggest that Nrf2 regulates the OA-NO_2 induced transcription of ET-B, which may lead to clearance of ET-1 from the circulation.

Materials and Methods

Reagents − OA-NO_2 was prepared as previously described (Woodcock *et al.*, 2013). The synthetic nitration product used in the study was an equimolar mixture of 9- and 10-nitro-octadec-9-enoic acid. BQ-788 was from Sigma (St. Louis, MO, USA).

Cell culture − Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from the maternity ward of the Kuopio University Hospital by the approval of the Kuopio University Hospital Ethics Committee. Each mother signed an informed consent. HUVECs were cultured as previously published (Levonen *et al.*, 2004). Cells from multiple donors were used for experiments at cell passages 4-6. Human aortic endothelial cells (HAECs) were obtained from Lonza (Bergisch Gladbach, Germany) and cultured as in (Kivelä *et al.*, 2010). Cells from a single donor were used at passages 8-10. Human aortic smooth muscle cells (HASMC) were purchased from Cascade Biologics (Portland, OR) and cultured in 231 medium supplemented with Smooth Muscle Cell Growth Supplement (Cascade Biologics, Portland, OR). Cells were from a single donor and used at passages 10-12. Mouse endothelial cells were isolated as described (Zhang *et al.*, 2009), with modifications. The lungs and hearts from 10 week-old wild type or Nrf2 knock out mice were removed under surgical anesthesia and collected in a tube containing cold base medium (DMEM with 20% FBS, 20 mM HEPES and 50 U/ml penicillin, 50 µg/ml streptomycin). Tissues were washed with 1xPBS and finely minced and digested using type II collagenase (Worthington Biochemical Corp, Lakewood, NJ) for 1 h at 37°C with gentle agitation. Digested tissues were passed through a 20-gauge needle 10–15 times and were then filtered through a 70-µm cell strainer. The digested filtrate was centrifuged, and the pellet was washed twice and resuspended in base

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medium. The filtrate was then incubated for 30 min at +4°C with 25 µg of anti-mouse CD31 antibody (BD Pharmingen, Minneapolis, MN) after which 100 µl of magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were added to the mixture and incubated at RT for 15 min. Cells with beads attached were collected using an MACS separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany) and washed 3 times with PBS. Washed cells were collected and plated in full medium (base medium with 1/100 NEAA, 1/100 Sodium Pyruvate, 1 mM L-glutamine, 0.1 mg/ml Endothelial mitogen, 0.1 mg/ml Heparin) tissue culture plates that had been precoated with 10 µg/ml Fibronectin (Sigma, St. Louis, MO, USA) in PBS. After 24h, nonattached cells and excess beads were removed, and fresh medium was added. Cells were further purified by repeating protocol with anti-mouse CD102 antibody (BD Pharmingen, Minneapolis, MN) and grown in full medium. For experiments, cells were cultured in EBM endothelial medium supplemented with EGM bullet kit (Lonza, Bergisch Gladbach, Germany). Cells were pooled from five mice and used for experiments at passages 4-6. Animal work was approved by National Experimental Animal Board of Finland and carried out following the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Western blot - HUVECs and HAECs were treated with 5 µM OA-NO_2 for 2 or 4h after which cells were collected to MNase buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 % NP-40, protease inhibitors) to extract the nuclear fraction. The extracted nuclei were lysed and the nuclear proteins were electrophoresed on a Tris/glycine SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The primary antibodies used for detection were rabbit polyclonal anti-Nrf2 (Santa Cruz Biotechnology), rabbit polyclonal anti-Lamin B1 (Abcam). Blots were visualized using Cy5-conjugated secondary antibodies with ChemiDoc (BioRad) scanner. Protein expression was quantified with ImageLab Software (Version 5.2.1 BioRad).

siRNA transfections − Small interfering RNA (siRNA) oligonucleotide targeting Nrf2 and a non-specific RNA control were obtained from Invitrogen (Carlsbad, CA). HUVECs or HAECs were seeded on 6-well plates at the density of 150 000 cells/well. Cells were allowed to adhere for

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24 h after which transfected with 50 nM siRNA oligonucleotides using Oligofectamine (Invitrogen, Carlsbad, CA). 24 h after transfection, cells were treated with OA-NO_2 for quantitative real-time PCR (qPCR).

Adenoviral overexpression – Cloning and production of AdNrf2 (Nrf2-overexpressing adenovirus) were performed as described previously (Levonen *et al.*, 2007). Multiplicity of infection (MOI) of 100 was used for experiments.

RNA isolation and qPCR − Cells were collected and RNA extracted with TRI Reagent (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. For the cDNA synthesis, 1 µg of total RNA was used using random hexamer primers (Promega, Madison WI) and Moloney-murine leukemia virus reverse transcriptase (Finnzymes, Espoo, Finland). The relative expression levels were measured according to the manufacturer's protocol with quantitative real time PCR (StepOnePlus™ Real-Time PCR systems, Applied Biosystems, Foster City, CA) using specific assays-on-demand (Applied Biosystems, Foster City, CA) target mixes. The expression levels were normalized to β2-microglobulin or to GAPDH expression and presented as fold change in the expression versus control.

Chromatin immonoprecipitation (ChIP) - . ChIP analysis was done as previously described (Kansanen *et al.*, 2011). Briefly, HUVECs were treated with 5 µM OA-NO_2 for 30 min to 2 h. Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 10 min at RT on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M for 5 min at RT on a rocking platform. Nuclei were extracted by scraping the cells to 1 ml of MNase buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM MgCl2, 0,1 % NP-40, protease inhibitors. The extracted nuclei were lysed with 0.3 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitors). The lysates were sonicated by a Bioruptor UCD-200 (Diagenode, Liege, y a Belgium) to result in DNA fragments of 200 to 1000 bp in length. Sonicated chromatin was divided in 100 µl aliquots and suspended in 1 ml of ChIP dilution buffer (0.01% SDS, 1.1%

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Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, protease inhibitors). 2.5 µl BSA (100 mg/ml) was added to each tube. 100 µl the chromatin sample was removed as input DNA and stored at + 4 C until Proteinase K treatment and purification. 100 µl of antibody-bound (Nrf2, sc-722, and anti-rabbit IgG, Sc-2027, Santa Cruz Biotechnologies) Magna ChIP magnetic beads (Millipore) were added to the chromatin samples and the samples were incubated O/N at + 4 C on a rocking platform. Next day, the beads were separated with a magnetic rack and washed five times with LiCl wash buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1 % IGEPAL, 1 % Sodium deoxycholate) and twice with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). For elution, 200 µl of elution buffer (1 % SDS, 0.1 M NaHCO3) was added to the beads and the mixture was incubated at RT for 1 h with vortexing the beads every 15 min. 100 µl of elution buffer was added to input sample. 2 µl of Proteinase-K (10 mg/ml, Thermo Scientific) was added to all samples and the samples were incubated at + 65 C O/N. Next day, DNA was purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Immunoprecipitated chromatin DNA was then used as a template for real-time quantitative PCR.

PCR of Chromatin Templates. Real-time quantitative PCR of ChIP templates was performed using specific primers for the *EDNRB* chromatin region 4665 (5'-TAGATGTGCAGAAGCCAGGA-3' and 5'-CACCTCCCGTTATCAGTTCTC-3'), or *EDNRB* chromatin region 5253 (5'-GGTGCGTTTGATGAACTGAA-3' and 5'-GAGAGCTGGTGGCTTCCATA-3'), or *HMOX1* chromatin region (5'-TGAGTAATCCTTTCCCGAGC-3' and 5'-GTGACTCAGCGAAAACAGACA-3') and FAST SYBR Green qPCR Master Mix in a total volume of 10 µl in a LightCycler 480 system (Roche Applied Science, Mannheim, Germany).

ET-1 ELISA. ET-1 concentration from cell culture medium was measured with Endothelin-1 Quantikine ELISA Kit (BD Biosciences, Minneapolis, MN) according to manufacturer's protocol.

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Statistical Analysis. Each experiment was performed at least in triplicate wells and repeated 2-5 times, and the representative experiment is presented. Statistical analysis was performed with GraphPad Prism (Version 5.03), and the data were analyzed by unpaired two tailed t-test analysis (t-test) for comparison between two groups, and one-way analysis of variance (ANOVA) with Tukey's post hoc comparison for multiple comparisons. Data are expressed as mean +/- SD, and differences were considered significant as follows: * $p< 0.05$; ** $p<0.01$, *** $p<0.001$. To calculate correlations, Pearson correlation test was applied.

Results

Previously, we studied Nrf2-dependent and independent effects of OA-NO_2 in human endothelial cells using a genome-wide expression analysis. The data indicated that OA-NO_2 upregulated ET-B receptor mRNA expression in an Nrf2-dependent manner (Kansanen *et al.*, 2009). To verify this finding, human umbilical vein endothelial cells (HUVEC) were treated with OA-NO_2 and the ET-B receptor mRNA expression was measured with quantitative PCR. *HMOX1* and *GCLM,* genes that are well known to be induced in response to OA-NO_2 treatment (Kansanen *et al.*, 2009), were used as a positive controls. OA-NO_2 increased both *HMOX1* (Figure 1A-C) and *GCLM* (Figure 1D-F) expression in a time and concentration dependent manner in HUVECs. When the expression of ET-B receptor was analyzed, the highest increase in expression after OA-NO_2 treatment was observed with 5 µM OA-NO_2. The ET-B receptor expression was increased 5.4-fold and 7.5-fold with 5 µM OA-NO_2 at 6 h and 16 h, respectively (Figure 1G-I).

The ET-B receptor is suggested to be the predominant receptor for ET-1 in endothelial cells. It mediates vasorelaxation and functions as a clearance receptor by removing ET-1 from the circulation. Vascular smooth muscle cells express both ET-A and ET-B receptors and activation of both receptors in these cells results in smooth muscle contraction (Schneider *et al.*, 2007). We compared the OA-NO_2 induced ET-B expression both in HUVECs and in human smooth

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muscle cells (HASMC) and found that the increase in ET-B expression evoked by OA-NO_2 was substantially lower in HASMCs than in HUVECs (Figure 2A). In HUVECs 2.5 μM OA-NO_2 induced ET-R receptor expression by 6.2-fold and 5 μM OA-NO_2 by 4.6-fold. In HASMCs the fold induction after 2.5 and 5 μM OA-NO_2 were 2.0 and 2.6, respectively. The difference between the fold changes shown in Figure 1 and Figure 2 is likely because of donor-specific differences in HUVEC isolations. Furthermore, OA-NO_2 did not increase the expression of ET-A receptor in HASMCs, and HUVECs did not express any detectable ET-A mRNA (Figure 2B). In comparison, OA-NO_2 induced the expression of Nrf2 target genes HMOX1 and GCLM in both HUVECs and HASMC. HMOX1 expression was higher in HASMC (Figure 2C), and there was no difference in GCLM mRNA expression when the two cell lines were compared (Figure 2D). To study whether the higher ET-B receptor expression in HUVECs was due to the higher expression of Nrf2, both mRNA expression and nuclear Nrf2 translocation were measured. Nrf2 mRNA expression was higher in HASMC, but the difference was significant only in basal condition (Figure 2E). As Nrf2 activation is mainly regulated at the post-transcriptional level (Suzuki and Yamamoto, 2015), nuclear translocation of Nrf2 after OA-NO_2 treatment was measured. OA-NO_2 increased the nuclear accumulation of Nrf2 in both cell lines, and the accumulation was more pronounced in HAECs (Figure 2F-G). Thus, the lower ET-B induction in response to OA-NO_2 is not due to lower Nrf2 expression, suggesting an alternative mechanism for more pronounced ET-B receptor expression in HUVECs.

To study whether Nrf2 mediates the OA-NO_2 induced upregulation of ET-B, the effect of Nrf2 overexpression was studied first. In HUVECs, overexpression of Nrf2 by adenovirus (AdNrf2, Figure 3A) resulted in a robust induction in ET-B mRNA (Figure 3B). Next, the role of Nrf2 silencing on the OA-NO_2 induced ET-B expression was examined in different cultured endothelial cells. A siRNA approach was used to silence Nrf2 in both human venous and aortic endothelial cells. In HUVECs and human aortic endothelial cells (HAECs), Nrf2-siRNA reduced Nrf2 expression 75% and 91% in basal and 70% and 93% in induced conditions, respectively (Figure 3C-D). Furthermore, ET-B expression was significantly reduced in HUVECs and

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HAECs in basal (44% and 77%) and OA-NO_2 induced (60% and 83%) conditions, respectively (Figure 3E-F). In addition, the role of Nrf2 in OA-NO_2 induced ET-B expression was studied in mouse endothelial cells isolated from wild type and Nrf2 knockout (Nrf2-KO) mouse hearts. Similar to human endothelial cells, a significant reduction in ET-B expression was detected in both basal (77%) and induced (83%) conditions (Figure 3G). These data show that in both human and mouse endothelial cells, Nrf2 is required for ET-B receptor mRNA expression.

Because ET-B receptor was expressed in an Nrf2-dependent manner in endothelial cells, we next studied whether ET-B is a direct target of Nrf2. Utilizing *in silico* screening for Nrf2 binding sites (Kuosmanen *et al.*, 2016), seven putative AREs were found at the vicinity of ET-B gene, *EDNRB* (Figure 4A). Two of the seven ARE sequences co-localized with ENCODE open chromatin markers (H3K4Me1 and H3K27Ac) and transcription factor (MafF, MafK, and BACH1) ChIP positions (Figure 4A). Nrf2 heterodimerizes with small Maf proteins to bind ARE sequences and BACH1 has been previously shown to bind AREs (Igarashi and Sun, 2006). These two AREs were located 4665 (Figure 4B) and 5253 (Figure 4C) base pairs from the gene transcription start site of the longest *ENDRB* transcript. To study whether Nrf2 binds to these sites in endothelial cells, a ChIP analysis was performed. The analysis revealed increased binding of Nrf2 to the ARE site located 5253 base pairs upstream from transcription start site 60 min after OA-NO_2 addition (Figure 4E). However, even though OA-NO_2 increased the binding of Nrf2 to the ARE site located 4665 base pairs from the transcription start, the binding remained lower than the background. (Figure 4D). The binding of Nrf2 to the distal enhancer region in *HMOX1* gene was used as a positive control (Kansanen *et al.*, 2011) (Figure 4F).

The function of the ET-B receptor is to mediate the vasodilatory effects of ET-1, and it also functions as a decoy receptor to clear ET-1 from the circulation (Kelland, Kuc, *et al.*, 2010). To study the functional effect of OA-NO_2 and Nrf2 induced ET-B expression, ET-1 peptide concentration was measured from the cell culture medium. OA-NO_2 was found to significantly decrease ET-1 concentration in the medium by 4h after addition of OA-NO_2 (Figure 5A). After

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24 h, the ET-1 concentration was reduced by 41% (Figure 5B). In addition, overexpression of Nrf2 (Figure 3A) decreased the amount of ET-1 detectable in the medium by 79% (Figure 5C), and Nrf2 silencing (Figure 3C) increased ET-1 concentration by 20% (Figure 5D). To investigate whether the change in extracellular ET-1 concentration was due to the transcriptional repression of ET-1 gene, *EDN1*, the mRNA expression of *EDN1* was measured. In contrast to ET-B receptor mRNA (Figure 5I-L), OA-NO_2 (Figure 5E-F), Nrf2 overexpression (Figure 5G) of Nrf2 silencing (Figure 5H) did not cause significant changes in *EDN1* gene expression. This data indicates that the changes in ET-1 concentration after OA-NO_2 treatment or Nrf2 modulation are not explained by transcriptional chances of *EDN1*, and rather correlate with the changes in ET-B receptor mRNA expression (Figure 5M-P).

Next, the role of ET-B receptor in OA-NO_2 and Nrf2 induced ET-1 clearance was investigated by using a specific ET-B antagonist BQ788. Even though BQ-788 did not change the extracellular ET-1 concentration in basal conditions, treatment with BQ-788 prior to the addition of OA-NO_2 abolished the reduction of ET-1 in the medium (Figure 6A). Similarly to OA-NO_2 treatment, ET-B receptor blockage with BQ-788 reversed the reduction of ET-1 concentration after Nrf2 overexpression (Figure 6B). Furthermore, Nrf2 silencing increased the amount of ET-1 in the cell culture medium (Figure 5H and Figure 6C). When ET-B receptor was blocked with BQ-788, the siNrf2-induced increase in ET-1 concentration was reduced from 2.2x to 1.7x, further confirming that OA-NO_2 and Nrf2 modulate the ET-B dependent clearance of ET-1 from the extracellular compartment.

Because ET-B receptor transcription is stringently regulated by Nrf2 (Figure 3C-E), the effect of Nrf2 silencing on OA-NO_2 induced ET-1 clearance was also examined. As expected, in non-treated conditions, Nrf2 silencing significantly increased and OA-NO_2 treatment decreased the amount of ET-1 by 61% in the cell culture medium (Figure 6D). However, when Nrf2 was silenced OA-NO_2 treatment decreased the ET-1 concentration even further (by 70%). At the same time, in control condition, siNrf2 increased the amount of ET-1 in the cell culture medium by 1.5x, but in OA-NO_2 treated cells, the increase was only 1.2x and did not reach statistical

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significance (Figure 6D). In addition to extracellular ET-1 concentration, ET-B receptor mRNA expression was measured from the samples. The changes in ET-1 concentration in the cell culture medium correlated with the changes in ET-B receptor expression (Figure 6I-J). When the extracellular ET-1 concentration was blotted against the ET-B receptor expression, there was a high (Figure 6I, K-L) to moderate (Figure 6J) correlation between the ET-1 concentrations and ET-B receptor expression. These data suggests that both OA-NO_2 and Nrf2 activation induce clearance of ET-1 via ET-B receptor.

Discussion

In this study, we show for the first time that OA-NO_2 modulates the endothelin signaling by inducing Nrf2-dependent expression of ET-B receptor, thereby decreasing extracellular ET-1 secreted by cultured endothelial cells. In addition, we show that Nrf2 directly regulates the ET-B receptor gene, *EDNRB*, and its expression is largely dependent on this transcription factor.

Nitro-fatty acids, such as OA-NO_2, are endogenous reactive lipids formed when unsaturated fatty acids react with nitric oxide or nitric oxide-derived species (Schopfer *et al.*, 2011). In vivo, nitro-fatty acids are measured at low nM concentrations but they are robustly elevated in inflammatory conditions (V Rudolph *et al.*, 2010; Salvatore *et al.*, 2013). The main mechanism and signaling action of OA-NO_2 is via post-transcriptional modification of regulatory proteins, such as PPARγ (Schopfer *et al.*, 2010), Keap1 (Kansanen *et al.*, 2011), and NF-κB (Cui *et al.*, 2006). Furthermore, OA-NO_2 can increase NO bioavailability via endothelial NO synthase phosphorylation (Khoo *et al.*, 2010). OA-NO_2 has shown to be beneficial in murine models of vascular disease (Cole *et al.*, 2009; TK Rudolph *et al.*, 2010), type 2 diabetes (Schopfer *et al.*, 2010), and both myocardial (V Rudolph *et al.*, 2010) and renal (Wang *et al.*, 2010) ischemia-reperfusion injury. Furthermore, in an Ang II-induced hypertension in mice, OA-NO_2 is shown to reduce blood pressure by direct adduction of the AT_1 receptor (Zhang *et al.*, 2010). In

addition, OA-NO_2 can inhibit the enzymatic activity of epoxyeicosatrienoic acid (EET) hydrolyzing soluble epoxide hydrolase by adduction to Cys521 in the vicinity of its catalytic center and this inhibition may mediate the antihypertensive effects of OA-NO_2 (Charles *et al.*, 2014). In this study, we show an additional potential mechanism by which OA-NO_2 may reduce blood pressure. This mechanism involves Nrf2-dependent increase in ET-B receptor expression, which leads to increased clearance of ET-1. In previous studies, we have shown that OA-NO_2 induces Nrf2-dependent activation via modification of Cys residues in Nrf2 inhibitor protein Keap1 (Kansanen *et al.*, 2011). Therefore, it can be postulated that the increase in Nrf2-dependent ET-B receptor expression is also mediated via post-translational modification of Keap1.

Endothelin-1 is a vasoactive 21 amino acid cyclic peptide, which was originally isolated from porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). Several cell types can synthesize and release ET-1, but the most important biological source is the endothelium. ET-1 has a half-life of less than 2 minutes in blood (Dhaun *et al.*, 2008) and it is rapidly taken up by the vasculature. The uptake involves binding of ET-1 to cell surface ET-B receptors, internalization of the ligand bound receptor, followed by receptor degradation, probably within lysosomes (Bremnes *et al.*, 2000). Endothelin receptors in different tissues regulate diverse physiological responses including vasoconstriction, vasodilation, clearance of ET-1, and renal sodium absorption (Schneider *et al.*, 2007; Kohan *et al.*, 2011). ET-1 has been shown to play a role in high salt-induced hypertension, likely via the combined effect of impaired ET-B receptor mediated ET-1 clearance as well as the activation of the ET-A receptor (Gariepy *et al.*, 2000; Pollock and Pollock, 2001; Amiri *et al.*, 2010). Therefore, the effects of selective ET-A, ET-B or dual ET-A/ET-B receptor antagonists on hypertension have been investigated. Results show that while ET-A or both ET-A and ET-B receptor inhibition with selective ET-A or dual ET-A/ET-B antagonists reduce blood pressure (Krum *et al.*, 1998; Nakov *et al.*, 2002), more profound effects are achieved with ET-B blockers, which increase blood pressure (Strachan *et al.*, 1999; Opgenorth *et al.*, 2000). These results suggest that the more important physiological role of

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ET-1 in systemic hypertension is through ET-B receptor actions that promote vasodilatation via preventing ET-A mediated constriction. A major drawback for ET receptor antagonists has been the high incidence of side effects including fluid retention, edema and hepatotoxicity, which largely prohibits their use in the general population to treat hypertension (Hoeper, 2009; Laffin and Bakris, 2015). Therefore, new treatment options are needed. In this study, both OA-NO_2 and Nrf2 overexpression induced the expression of the ET-B receptor expression in endothelial cells, and decreased the amount of ET-1 in cell culture medium. In addition, silencing of Nrf2 decreased ET-B receptor expression and increased the ET-1 concentration in cell culture medium. Our data suggests that Nrf2 and Nrf2 inducing agents, via promoting the clearance of ET-1 by ET-B receptor, may reduce circulating ET-1 levels thereby limiting its vasoconstrictive effects. In our study, the ET-B receptor antagonist BQ-788 did not change the extracellular concentration of ET-1 in basal conditions, but reversed the reduction in ET-1 concentration after OA-NO_2 treatment or Nrf2 modulation. Because BQ-788 cannot displace the bound ET-1 from the ET-B receptor (Johnström et al., 2005), we suggest that the change in ET-B receptor expression was needed for the change in the extracellular concentration of ET-1. Our results are consistent with the findings that OA-NO_2 has anti-hypertensive effects in mouse models of hypertension (Zhang *et al.*, 2010; Charles *et al.*, 2014). Furthermore, increasing or restoring ET-B receptor function may be helpful also in other diseases where ET-1 production is increased, such as chronic kidney disease (Cottone *et al.*, 2009) and pulmonary arterial hypertension (PAH) (McLaughlin, 2006).

Current clinical use of ET receptor antagonists is limited to pulmonary arterial hypertension (PAH). PAH is a progressive disease characterized by the elevation of pulmonary artery pressure and adverse vascular remodeling leading to right ventricular dysfunction. PAH has a poor prognosis and limited treatment options (McLaughlin, 2006). Endothelial ET-B receptor function is important in limiting the development of PAH in response to hypoxia (Kelland, Bagnall, *et al.*, 2010). ET-1 levels are elevated in patients with PAH, and the clearance of ET-1 in the pulmonary vasculature is reduced. Plasma levels of ET-1 correlate with the severity of

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PAH (McLaughlin, 2006). Previously, the effect of OA-NO_2 on PAH has been studied using the hypoxia-induced mouse model (Klinke *et al.*, 2014). OA-NO_2 reversed the development of PAH and consequent right ventricular dysfunction. The protective effect of OA-NO_2 was linked to a decrease in oxidative inflammatory responses in pulmonary smooth muscle cells and macrophages. OA-NO_2 inhibited pulmonary smooth muscle cell proliferation and reduced right ventricular remodeling (Klinke *et al.*, 2014). Furthermore, in obesity-induced model of PAH, treatment with OA-NO_2 improved right ventricular function (Kelley *et al.*, 2014). Our data suggests that in addition to the effects on pulmonary smooth muscle cells (Klinke *et al.*, 2014), the beneficial effect of OA-NO_2 in PAH may be related to the regulation of endothelin system, as OA-NO_2 increases the clearance of ET-1 via ET-B receptor upregulation. Interestingly, another study by Eba et al. showed that mice deficient in Nrf2 inhibiting protein Keap1 that have a sustained increase in Nrf2 activity are protected against hypoxia-induced pulmonary alterations related to PAH, whereas these were aggravated in Nrf2-deficient mice (Eba *et al.*, 2013). Similar to genetic overexpression, the Nrf2 inducer oltipraz afforded protection against pulmonary artery muscularization in wild type but not in Nrf2-deficient mice (Eba *et al.*, 2013), highlighting the therapeutic potential of Nrf2 activators in the treatment of PAH.

Our results suggest that there is a cell type specific difference in the regulation of the ET-B receptor mRNA in HUVECs and HASMCs. In contrast to Nrf2 target gene and Nrf2 mRNA expression, OA-NO_2 had a substantially smaller effect on ET-B receptor mRNA expression in HASMC than in HUVECs. As cell type specific gene regulation is largely regulated by epigenetic mechanisms, the different response in these cells lines may be due to difference in methylation of gene regulatory regions. Methylation of these regions renders chromatin inaccessible to binding of a given transcription factor in one cell type whereas the chromatin is maintained in an open conformation allowing transcription factor binding in another cell type (Shirodkar *et al.*, 2013). The ET-B receptors in endothelial cells function to maintain appropriate plasma level of ET-1, and the function of ET-B receptors in other cell types such

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as smooth muscle cells is less clear, which may explain the cell type specific difference in the ET-B receptor expression.

To conclude, we have shown that Nrf2 regulates OA-NO_2 induced transcription of ET-B in vascular endothelial cells. The effect of OA-NO_2 did not change the expression of the ET-A receptor and had a substantially smaller effect on ET-B receptor expression in smooth muscle cells. Furthermore, we show that both OA-NO_2 and Nrf2 regulate the ET-B dependent clearance of ET-1 in endothelial cells. Therefore, we suggest that OA-NO_2 may alleviate vasoconstrictive effects of ET-1 by removing it from the circulation, thus potentially affecting blood pressure regulation.

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Authorship Contributions

Participated in research design: Kansanen, Levonen.

Conducted experiments: Kansanen, Kuosmanen, Ruotsalainen, Hynynen

Performed data analysis: Kansanen

Wrote or contributed to the writing of the manuscript: Kansanen, Kuosmanen, Ruotsalainen, Levonen

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References

Amiri F, Ko E a, Javeshghani D, Reudelhuber TL, and Schiffrin EL (2010) Deleterious combined effects of salt-loading and endothelial cell restricted endothelin-1 overexpression on blood pressure and vascular function in mice. *J Hypertens* **28**:1243–51.

Baker LMS, Baker PRS, Golin-Bisello F, Schopfer FJ, Fink M, Woodcock SR, Branchaud BP, Radi R, and Freeman B a (2007) Nitro-fatty acid reaction with glutathione and cysteine. Kinetic analysis of thiol alkylation by a Michael addition reaction. *J Biol Chem* **282**:31085–93.

Batthyany C, Schopfer FJ, Baker PRS, Durán R, Baker LMS, Huang Y, Cerveñansky C, Branchaud BP, and Freeman B a. (2006) Reversible post-translational modification of proteins by nitrated fatty acids in vivo. *J Biol Chem* **281**:20450–20463.

Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, and Attramadal H (2000) Regulation and Intracellular Trafficking Pathways of the Endothelin Receptors. *J Biol Chem* **275**:17596–17604.

Charles RL, Rudyk O, Prysyazhna O, Kamynina A, Yang J, Morisseau C, Hammock BD, Freeman B a, and Eaton P (2014) Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase. *Proc Natl Acad Sci U S A* **111**:8167–72.

Cole MP, Rudolph TK, Khoo NKH, Motanya UN, Golin-Bisello F, Wertz JW, Schopfer FJ, Rudolph V, Woodcock SR, Bolisetty S, Ali MS, Zhang J, Chen YE, Agarwal A, Freeman B a., and Bauer PM (2009) Nitro-Fatty Acid Inhibition of Neointima Formation After Endoluminal Vessel Injury. *Circ Res* **105**:965–972.

Cottone S, Mulè G, Guarneri M, Palermo A, Lorito MC, Riccobene R, Arsena R, Vaccaro F, Vadalà A, Nardi E, Cusimano P, and Cerasola G (2009) Endothelin-1 and F2-isoprostane relate to and predict renal dysfunction in hypertensive patients. *Nephrol Dial Transplant* **24**:497–503.

Cui T, Schopfer FJ, Zhang J, Chen K, Ichikawa T, Baker PRS, Batthyany C, Chacko BK, Feng X, Patel RP, Agarwal A, Freeman B a, and Chen YE (2006) Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. *J Biol Chem* **281**:35686–98.

Dhaun N, Goddard J, Kohan DE, Pollock DM, Schiffrin EL, and Webb DJ (2008) Role of endothelin-1 in clinical hypertension: 20 years on. *Hypertension* **52**:452–459.

Eba S, Hoshikawa Y, Moriguchi T, Mitsuishi Y, Satoh H, Ishida K, Watanabe T, Shimizu T, Shimokawa H, Okada Y, Yamamoto M, and Kondo T (2013) The nuclear factor erythroid 2-related factor 2 activator oltipraz attenuates chronic hypoxia-induced cardiopulmonary alterations in mice. *Am J Respir Cell Mol Biol* **49**:324–33.

Gariepy CE, Ohuchi T, Williams SC, Richardson JA, and Yanagisawa M (2000) Salt-sensitive hypertension in endothelin-B receptor-deficient rats. *J Clin Invest* **105**:925–33.

Hoeper MMM (2009) Liver toxicity: the Achilles' heel of endothelin receptor antagonist therapy? *Eur Respir J Off J Eur Soc Clin Respir Physiol* **34**:529–530.

Igarashi K, and Sun J (2006) The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. *Antioxid Redox Signal* **8**:107–118.

Kansanen E, Bonacci G, Schopfer FJ, Kuosmanen SM, Tong KI, Leinonen H, Woodcock SR, Yamamoto M, Carlberg C, Ylä-Herttuala S, Freeman B a, and Levonen A-L (2011) Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism. *J Biol Chem* **286**:14019–27.

Kansanen E, Jyrkkanen H-K, Volger OL, Leinonen H, Kivela a. M, Hakkinen S-K, Woodcock SR, Schopfer FJ, Horrevoets a. J, Yla-Herttuala S, Freeman B a., and Levonen A-L (2009) Nrf2-dependent and -independent Responses to Nitro-fatty Acids in Human Endothelial Cells: identification of heat shock response as the major pathway activated by nitro-oleic acid. *J Biol Chem* **284**:33233–33241.

Kansanen E, Jyrkkänen HK, and Levonen AL (2012) Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic Biol Med* **52**:973–982.

Kelland NF, Bagnall AJ, Morecroft I, Gulliver-Sloan FH, Dempsie Y, Nilsen M, Yanagisawa M, MacLean MR, Kotelevtsev YV, and Webb DJ (2010) Endothelial ETB Limits Vascular Remodelling and Development of Pulmonary Hypertension during Hypoxia. *J Vasc Res* **47**:16–22.

Kelland NF, Kuc RE, McLean DL, Azfer A, Bagnall AJ, Gray G a., Gulliver-Sloan FH, Maguire JJ, Davenport AP, Kotelevtsev YV, and Webb DJ (2010) Endothelial cell-specific ET B receptor knockout: autoradiographic and histological characterisation and crucial role in the clearance of endothelin-1. *Can J Physiol Pharmacol* **88**:644–651.

Kelley EE, Baust J, Bonacci G, Golin-Bisello F, Devlin JE, St. Croix CM, Watkins SC, Gor S, Cantu-Medellin N, Weidert ER, Frisbee JC, Gladwin MT, Champion HC, Freeman BA, and Khoo NKH (2014) Fatty acid nitroalkenes ameliorate glucose intolerance and pulmonary hypertension in high-fat diet-induced obesity. *Cardiovasc Res* **101**:352–363.

Khoo NKH, Rudolph V, Cole MP, Golin-Bisello F, Schopfer FJ, Woodcock SR, Batthyany C, and Freeman B a (2010) Activation of vascular endothelial nitric oxide synthase and heme oxygenase-1 expression by electrophilic nitro-fatty acids. *Free Radic Biol Med* **48**:230–9.

Kivelä AM, Mäkinen PI, Jyrkkänen H-K, Mella-Aho E, Xia Y, Kansanen E, Leinonen H, Verma IM, Ylä-Herttuala S, and Levonen A-L (2010) Sulforaphane inhibits endothelial lipase expression through NF-κB in endothelial cells. *Atherosclerosis* **213**:122–8.

Klinke A, Möller A, Pekarova M, Ravekes T, Friedrichs K, Berlin M, Scheu KM, Kubala L, Kolarova H, Ambrozova G, Schermuly RT, Woodcock SR, Freeman B a, Rosenkranz S, Baldus S, Rudolph V, and Rudolph TK (2014) Protective effects of 10-nitro-oleic acid in a hypoxia-induced murine model of pulmonary hypertension. *Am J Respir Cell Mol Biol* **51**:155–62.

Kohan DE, Rossi NF, Inscho EW, and Pollock DM (2011) Regulation of blood pressure and salt homeostasis by endothelin. *Physiol Rev* **91**:1–77.

Krum H, Viskoper RJ, Lacourciere Y, Budde M, and Charlon V (1998) The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. *N Engl J Med* **338**:784–790.

Kuosmanen SM, Viitala S, Laitinen T, Peräkylä M, Pölönen P, Kansanen E, Leinonen H, Raju S, Wienecke-Baldacchino A, Närvänen A, Poso A, Heinäniemi M, Heikkinen S, and Levonen AL (2016) The Effects of Sequence Variation on Genome-wide NRF2 Binding - New Target Genes and Regulatory SNPs. *Nucleic Acids Res* **44**:1760–1775.

Kwak M-K, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, and Kensler TW (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. *J Biol Chem* **278**:8135–8145.

Laffin LJ, and Bakris GL (2015) Endothelin Antagonism and Hypertension: An Evolving Target. *Semin Nephrol* **35**:168–175.

Lee T-S, Tsai H-L, and Chau L-Y (2003) Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-Delta 12,14-prostaglandin J2. *J Biol Chem* **278**:19325–30.

Levonen AL, Inkala M, Heikura T, Jauhiainen S, Jyrkkänen HK, Kansanen E, Määttä K, Romppanen E, Turunen P, Rutanen J, and Ylä-Herttuala S (2007) Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo. *Arterioscler Thromb Vasc Biol* **27**:741–747.

Levonen A-L, Landar A, Ramachandran A, Ceaser EK, Dickinson D a, Zanoni G, Morrow JD, and Darley-Usmar VM (2004) Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem J* **378**:373–382.

McLaughlin V V. (2006) Pulmonary Arterial Hypertension. *Circulation* **114**:1417–1431.

Nadtochiy SM, Baker PRS, Freeman B a., and Brookes PS (2009) Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: Implications for cardioprotection. *Cardiovasc Res* **82**:333–340.

Nakov R, Pfarr E, and Eberle S (2002) Darusentan: an effective endothelinA receptor antagonist for treatment of hypertension. *Am J Hypertens* **15**:583–9.

Opgenorth TJ, Wessale JL, Dixon DB, Adler a L, Calzadilla S V, Padley RJ, and Wu-Wong JR (2000) Effects of endothelin receptor antagonists on the plasma immunoreactive endothelin-1 level. *J Cardiovasc Pharmacol* **36**:S292–6.

Pollock DM, and Pollock JS (2001) Evidence for endothelin involvement in the response to high salt. *Am J Physiol Renal Physiol* **281**:F144–50.

Rudolph TK, Rudolph V, Edreira MM, Cole MP, Bonacci G, Schopfer FJ, Woodcock SR, Franek A, Pekarova M, Khoo NKH, Hasty AH, Baldus S, and Freeman B a. (2010) Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **30**:938–945.

Rudolph V, Rudolph TK, Schopfer FJ, Bonacci G, Woodcock SR, Cole MP, Baker PRS, Ramani R, and Freeman B a. (2010) Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovasc Res* **85**:155–166.

Salvatore SR, Vitturi D a, Baker PRS, Bonacci G, Koenitzer JR, Woodcock SR, Freeman B a, and Schopfer FJ (2013) Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine. *J Lipid Res* **54**:1998–2009.

Schneider MP, Boesen EI, and Pollock DM (2007) Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. *Annu Rev Pharmacol Toxicol* **47**:731–59.

Schopfer FJ, Cipollina C, and Freeman B a (2011) Formation and Signaling Actions of Electrophilic Lipids. *Chem Rev* **111**:5997–6021.

Schopfer FJ, Cole MP, Groeger AL, Chen CS, Khoo NKH, Woodcock SR, Golin-Bisello F, Nkiru Motanya U, Li Y, Zhang J, Garcia-Barrio MT, Rudolph TK, Rudolph V, Bonacci G, Baker PRS, Xu HE, Batthyany CI, Chen YE, Hallis TM, and Freeman B a. (2010) Covalent peroxisome proliferator-activated receptor γ adduction by nitro-fatty acids: Selective ligand activity and anti-diabetic signaling actions. *J Biol Chem* **285**:12321–12333.

Shirodkar A V., St Bernard R, Gavryushova A, Kop A, Knight BJ, Yan MSC, Man HSJ, Sud M, Hebbel RP, Oettgen P, Aird WC, and Marsden PA (2013) A mechanistic role for DNA

methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing. *Blood* **121**:3531–3540.

Strachan FE, Spratt JC, Wilkinson IB, Johnston NR, Gray G a, and Webb DJ (1999) Systemic blockade of the endothelin-B receptor increases peripheral vascular resistance in healthy men. *Hypertension* **33**:581–5.

Suzuki T, and Yamamoto M (2015) Molecular basis of the Keap1-Nrf2 system. *Free Radic Biol Med* **88**:93–100.

Wang H, Liu H, Jia Z, Olsen C, Litwin S, Guan G, and Yang T (2010) Nitro-oleic acid protects against endotoxin-induced endotoxemia and multiorgan injury in mice. *Am J Physiol Renal Physiol* **298**:F754–F762.

Woodcock SR, Bonacci G, Gelhaus SL, and Schopfer FJ (2013) Nitrated fatty acids: Synthesis and measurement. *Free Radic Biol Med* **59**:14–26.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**:411–415.

Zhang DD, and Hannink M (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* **23**:8137–8151.

Zhang J, Villacorta L, Chang L, Fan Z, Hamblin M, Zhu T, Chen CS, Cole MP, Schopfer FJ, Deng CX, Garcia-Barrio MT, Feng Y-H, Freeman B a, and Chen YE (2010) Nitro-oleic acid inhibits angiotensin II-induced hypertension. *Circ Res* **107**:540–8.

Zhang X, Kazerounian S, Duquette M, Perruzzi C, Nagy J a, Dvorak HF, Parangi S, and Lawler J (2009) Thrombospondin-1 modulates vascular endothelial growth factor activity at the receptor level. *FASEB J* **23**:3368–3376.

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Figure Legends.

Figure 1. OA-NO_2 increases the expression of ET-B receptor in endothelial cells. A-I. HUVECs were treated with indicated times and concentrations of OA-NO_2 after which expression of HMOX1 **(A-C)**, GCLM **(D-F)** and ET-B **(G-I)** was measured with qPCR. Values are presented as mean +/- SD, n=3 ** p<0.01, *** p<0.001 versus control. ANOVA (A-I).

Figure 2. Differential expression of ET-A receptor, ET-B receptor in endothelial and smooth muscle cells. A-E. HUVECs or HASMCs were treated with indicated concentrations of OA-NO_2 for 8h. The expressions of ET-B receptor **(A)**, ET-A receptor **(B)**, HMOX1 **(C)**, GCLM **(D)** and Nrf2 **(E)** were measured with qPCR. Values are presented as mean +/- SD, n=3 ** p<0.01, *** p<0.001 versus control. Nd, not detected. ANOVA (A-C). **F**. HUVECs or HASMCs were treated with 5 µM OA-NO_2 for 2 and 4h. Nuclear extracts were isolated, and Nrf2 expression was analyzed by Western blot. Lamin B1 was used as control for nuclear extracts. **G.** The bar graph depicts the densitometric results of Nrf2 expression in nuclear fractions relative to LaminB1.

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Figure 3. Overexpression of Nrf2 increases and silencing or absence of Nrf2 decreases ET-B receptor expression in endothelial cells. A - B. HUVECs were transduced with control (AdCMV) Nrf2 overexpressing (AdNrf2) adenovirus and the expression of Nrf2 **(A)** and ET-B **(B)** was measured 48h after transduction. **C - D.** HUVECs and HAECs were transfected with control or Nrf2 siRNA and 24h after transfection, cells were treated with vehicle or 3 µM OA-NO_2 for 8 h. **F.** Endothelial cells isolated from wild type (WT) of Nrf2-KO mouse hearts (mEC) were treated with 3 µM OA-NO_2 for 8 h. The expression of Nrf2 **(C-D)** and ET-B **(E-G)** was determined with qPCR. Values are presented as mean +/- SD, A-B, n=9; C-G, n=3; ** $p<0.01$, *** $p<0.001$ versus respective control. ANOVA (C-G), t-test (A-B).

Figure 4. OA-NO_2 induces the binding of Nrf2 to ARE sequence located on the active enhancer region at the ET-B gene, *EDNRB*. A. ARE prediction found 7 ARE sequences from the enhancer region of *EDNRD*. Two of the AREs co-localized with ENCODE Txn Factor ChIP positions. **B - C.** Detailed view showing ARE located 4665 **(B)** or 5253 **(C)** bp from the gene transcription start site of the longest *ENDRB* transcript. **D-F.** HUVECs were treated with indicated times with 5 µM OA-NO_2 and the binding of Nrf2 to AREs located in *EDNRB* **(D-F)** or *HMOX1* **(F)** enhancer were analyzed with ChIP. Values are presented as mean +/- SEM, n=3), ** $p<0.01$, *** $p<0.001$ versus control. Txn Factor ChIP-seq track displays combined MafF, MafK, and BACH1 binding signals in H1-hESC, HepG2 and IMR90 cell lines. H3K4Me1 and H3K27Ac tracks mark active chromatin regions in HUVECs. ANOVA (D-E).

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Figure 5. OA-NO_2 and Nrf2 modulates ET-1 concentration in HUVEC medium but do not change EDN1 expression. A-L. HUVECs were treated with 5 µM OA-NO2 for 4h or 24h, transduced with control (AdCMV) or Nrf2 overexpressing (AdNrf2) adenovirus or transfected with control (siCtrl) or Nrf2 (siNrf2) siRNA. Cells and cell culture medium was collected for analysis 4h or 24h after OA-NO_2 treatment. For adenoviral transductions or siRNA transfections, cell culture medium was changed 24h after transduction of transfection and cells and cell culture medium was collected for analysis 24h after that. ET-1 concentration from the cell culture media was measured with ELISA **(A-D),** and EDN1 **(E-H),** and ET-B receptor **(I-L)** expression from the cells with qPCR. Values are presented as mean +/- SD, n=3 * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus respective control. t-test (A-L). **M-P.** ET-1 levels and ET-B receptor expression was plotted against each other for each experimental setting. Correlation was determined using Pearson correlation coefficient, and r2 and p values are shown.

Figure. 6. OA-NO_2 and Nrf2 induced ET-1 clearance is dependent on ET-B receptor. **A, E.** HUVECs were treated with ET-B receptor antagonist 1 µM BQ-788 for 8h, after which 5 µM OA-NO_2 was added for additional 16h. **B, F**. HUVECs were transduced with control (AdCMV) or Nrf2 (AdNrf2) overexpressing adenovirus. 24 h after transduction cells were treated with 1 µM BQ-788 for 24h. **C, G.** HUVECs were transfected with control or Nrf2 siRNA and 24h after transfection, cells were treated with 1 µM BQ-788 for 24 h. **D, H.** HUVECs were transfected with control or Nrf2 siRNA and 24h after transfection, cells were treated with 5 µM OA-NO_2 for 16 h**.** ET-1 concentration in cell culture medium was measured with ELISA **(A-D),** and ET-B mRNA expression with qPCR **(E-H).** Values are presented as mean +/- SD (A, C-E, G-H, n=3; B, F, n=6), * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ANOVA (A-H). **I-L.** ET-1 levels and ET-B receptor expression was plotted against each other for each experimental setting. Correlation was determined using Pearson correlation coefficient, and r^2 and p values are shown.

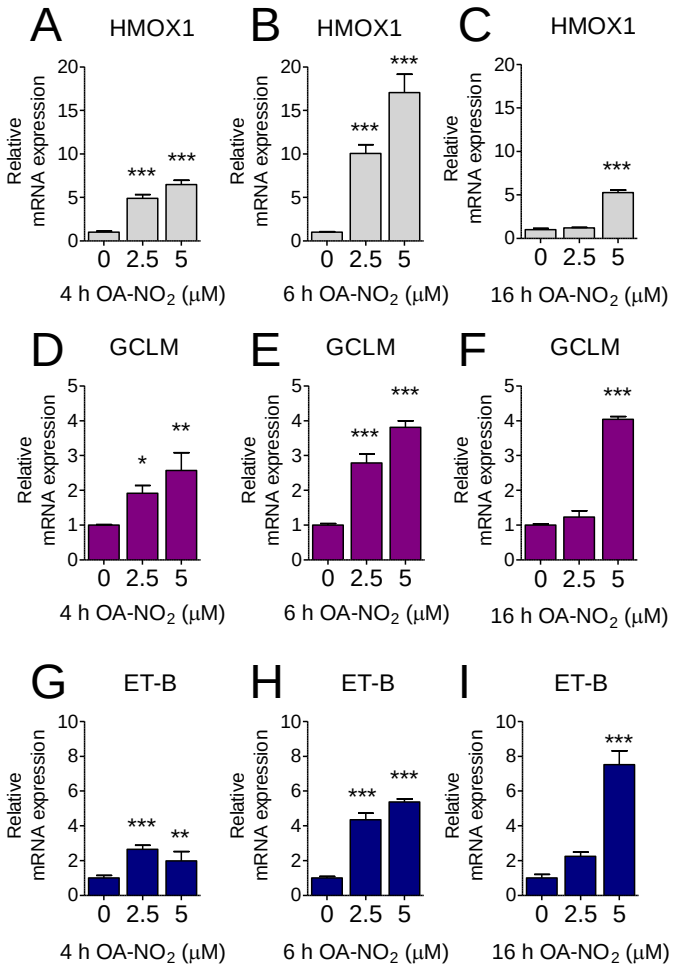
A
HMOX1
B
HMOX1
C
HMOX1
Relative mRNA expression
0 2.5 5
4 h OA-NO2 (μM)
6 h OA-NO2 (μM)
16 h OA-NO2 (μM)
D
GCLM
E
GCLM
F
GCLM
G
ET-B
H
ET-B
I
ET-B

Figure 1

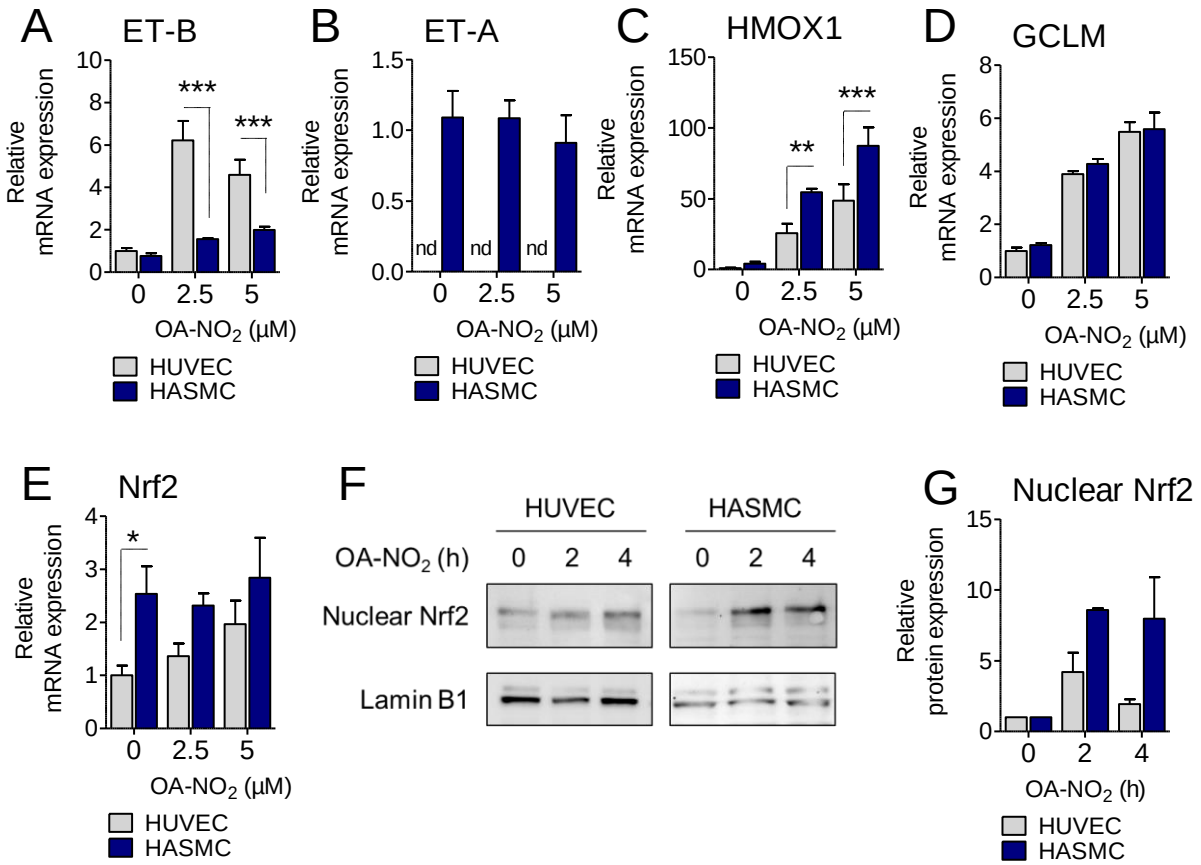
A
ET-B
Relative mRNA expression
***
***
OA-NO2 (μM)
HUVEC
HASMC
B
ET-A
nd
nd
nd
C
HMOX1
**
***
D
GCLM
E
Nrf2
*
F
HUVEC
HASMC
OA-NO2 (h)
Nuclear Nrf2
Lamin B1
G
Nuclear Nrf2
Relative protein expression

Figure 2

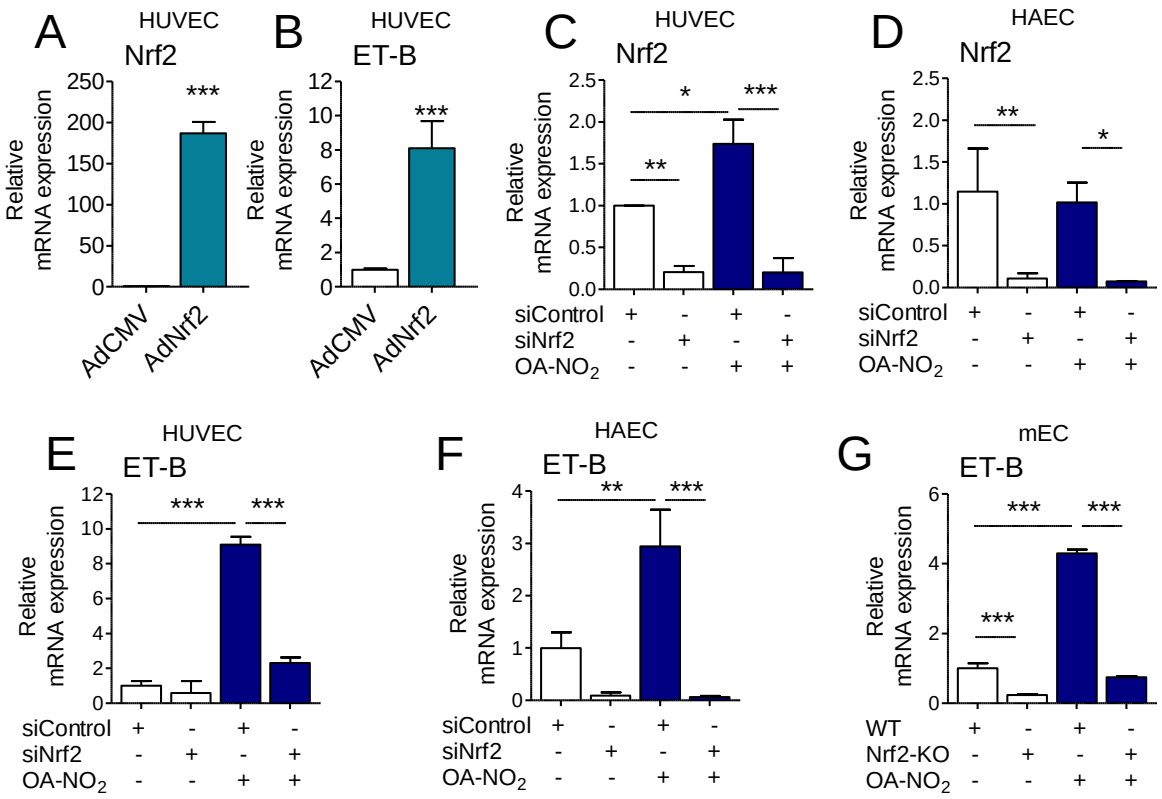
A
HUVEC
Nrf2
Relative mRNA expression
250
200
150
100
50
0
***
AdCMV
AdNrf2
B
HUVEC
ET-B
Relative mRNA expression
12
10
8
6
4
2
0
***
AdCMV
AdNrf2
C
HUVEC
Nrf2
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
***
**
siControl + - + -
siNrf2 - + - +
OA-NO2 - - + +
D
HAEC
Nrf2
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
**
*
siControl + - + -
siNrf2 - + - +
OA-NO2 - - + +
E
HUVEC
ET-B
Relative mRNA expression
12
10
8
6
4
2
0
***
***
siControl + - + -
siNrf2 - + - +
OA-NO2 - - + +
F
HAEC
ET-B
Relative mRNA expression
4
3
2
1
0
**
***
siControl + - + -
siNrf2 - + - +
OA-NO2 - - + +
G
mEC
ET-B
Relative mRNA expression
6
4
2
0
***
***
***
WT + - + -
Nrf2-KO - + - +
OA-NO2 - - + +

Figure 3

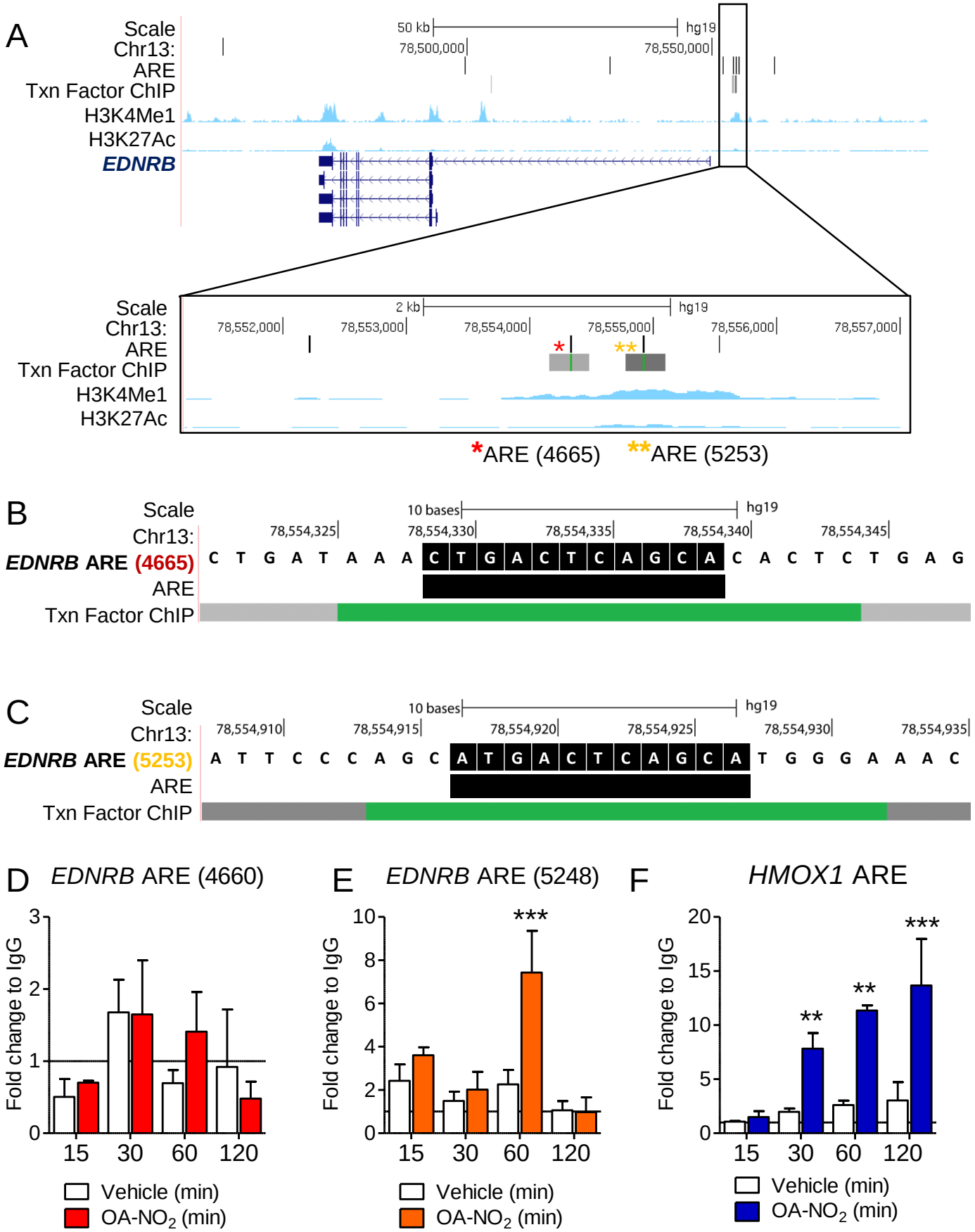
A
Scale
Chr13:
ARE
Txn Factor ChIP
H3K4Me1
H3K27Ac
EDNRB
50 kb
hg19
78,500,000
78,550,000
2 kb
78,552,000
78,553,000
78,554,000
78,555,000
78,556,000
78,557,000
*ARE (4665)
**ARE (5253)
B
EDNRB ARE (4665)
10 bases
78,554,325
78,554,330
78,554,335
78,554,340
78,554,345
C T G A T A A A C T G A C T C A G C A C A C T C T G A G
C
EDNRB ARE (5253)
78,554,910
78,554,915
78,554,920
78,554,925
78,554,930
78,554,935
A T T C C C A G C A T G A C T C A G C A T G G G A A A C
D
EDNRB ARE (4660)
Fold change to IgG
Vehicle (min)
OA-NO2 (min)
E
EDNRB ARE (5248)
F
HMOX1 ARE
15
30
60
120

Figure 4

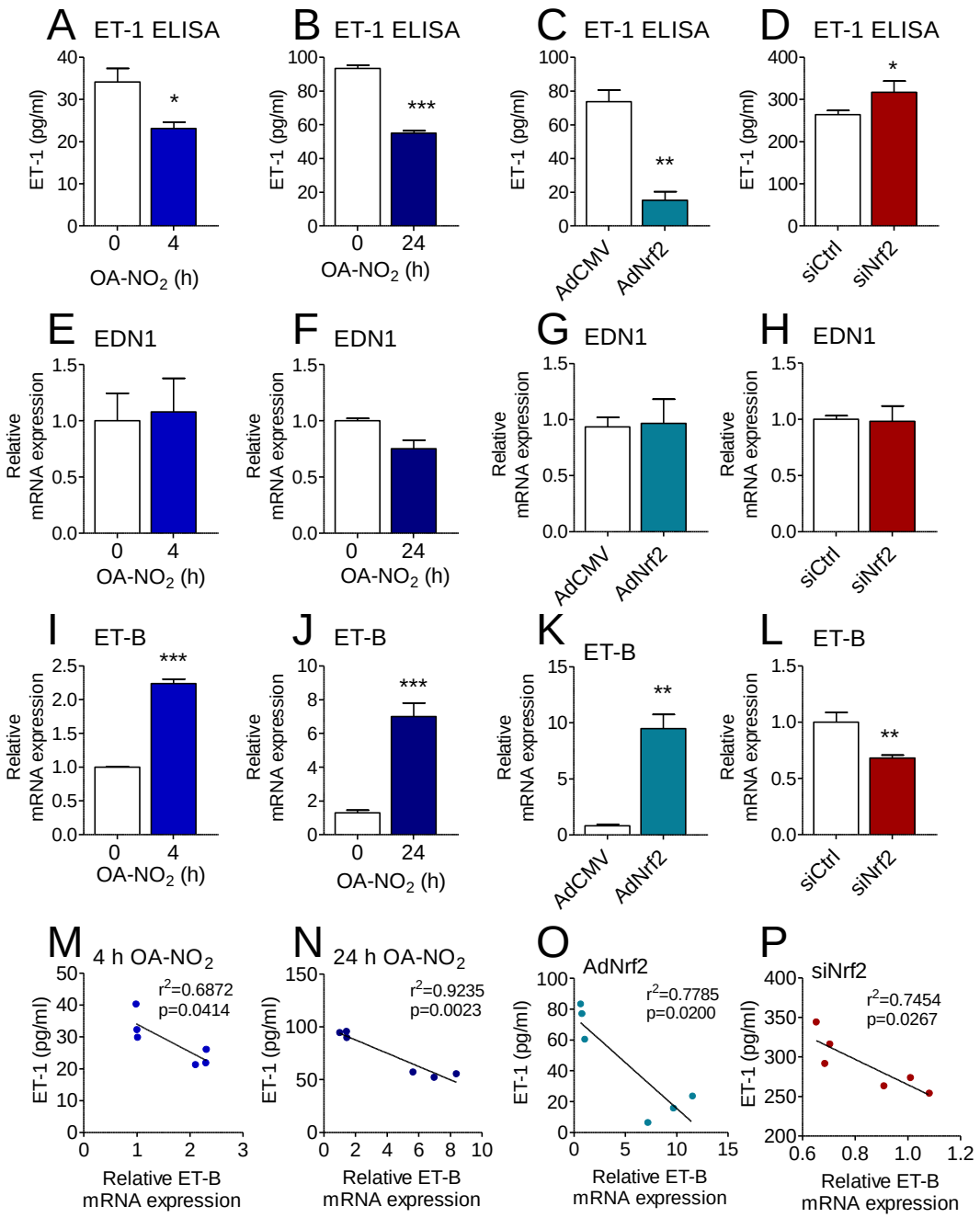
A ET-1 ELISA
ET-1 (pg/ml)
OA-NO2 (h)
B ET-1 ELISA
ET-1 (pg/ml)
OA-NO2 (h)
C ET-1 ELISA
ET-1 (pg/ml)
AdCMV
AdNrf2
D ET-1 ELISA
ET-1 (pg/ml)
siCtrl
siNrf2
E EDN1
Relative mRNA expression
OA-NO2 (h)
F EDN1
Relative mRNA expression
OA-NO2 (h)
G EDN1
Relative mRNA expression
AdCMV
AdNrf2
H EDN1
Relative mRNA expression
siCtrl
siNrf2
I ET-B
Relative mRNA expression
OA-NO2 (h)
J ET-B
Relative mRNA expression
OA-NO2 (h)
K ET-B
Relative mRNA expression
AdCMV
AdNrf2
L ET-B
Relative mRNA expression
siCtrl
siNrf2
M 4 h OA-NO2
r2=0.6872
p=0.0414
ET-1 (pg/ml)
Relative ET-B mRNA expression
N 24 h OA-NO2
r2=0.9235
p=0.0023
ET-1 (pg/ml)
Relative ET-B mRNA expression
O AdNrf2
r2=0.7785
p=0.0200
ET-1 (pg/ml)
Relative ET-B mRNA expression
P siNrf2
r2=0.7454
p=0.0267
ET-1 (pg/ml)
Relative ET-B mRNA expression

Figure 5

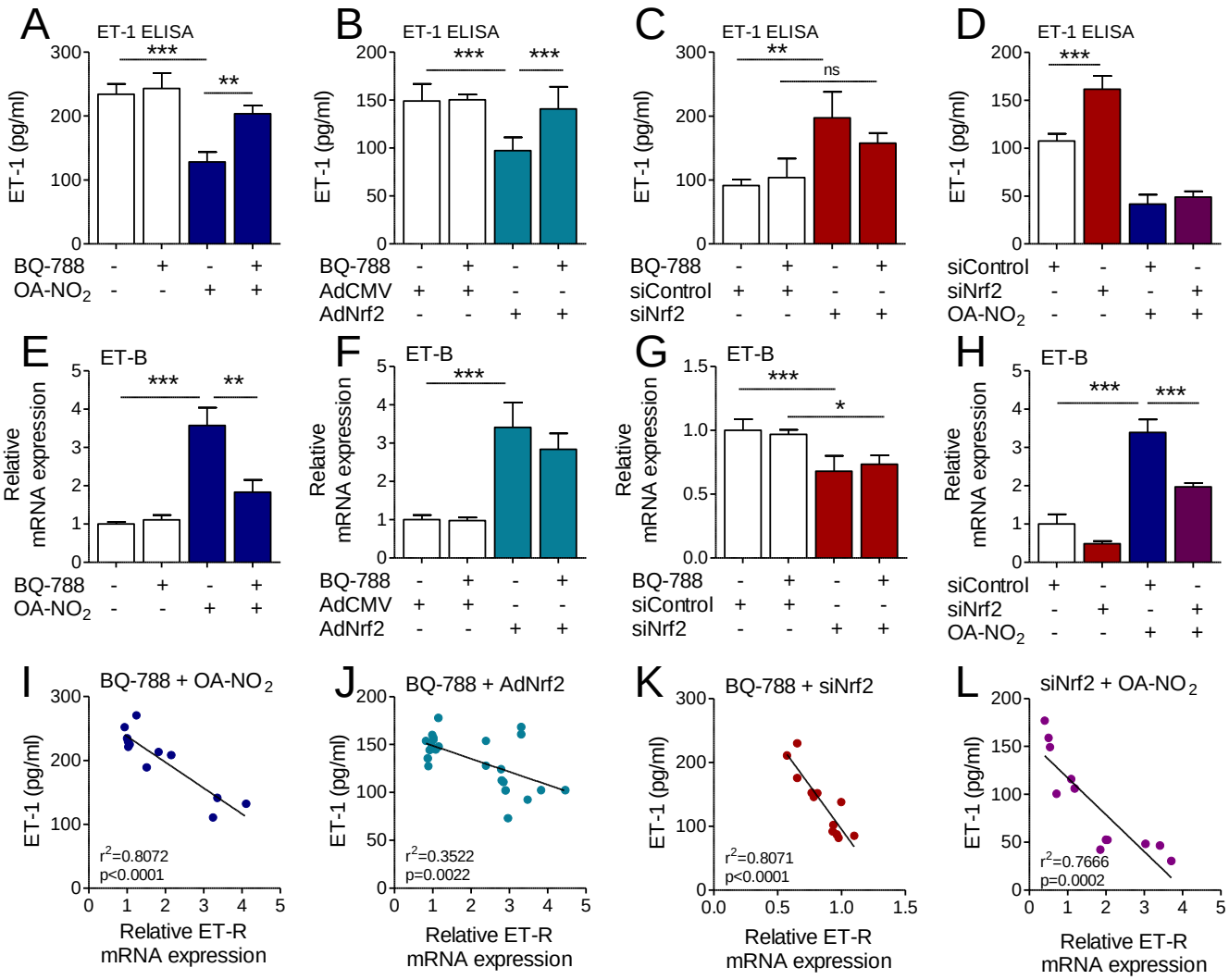
A
ET-1 ELISA
ET-1 (pg/ml)
BQ-788
OA-NO2
B
ET-1 ELISA
ET-1 (pg/ml)
BQ-788
AdCMV
AdNrf2
C
ET-1 ELISA
ET-1 (pg/ml)
BQ-788
siControl
siNrf2
ns
D
ET-1 ELISA
ET-1 (pg/ml)
siControl
siNrf2
OA-NO2
E
ET-B
Relative mRNA expression
BQ-788
OA-NO2
F
ET-B
Relative mRNA expression
BQ-788
AdCMV
AdNrf2
G
ET-B
Relative mRNA expression
BQ-788
siControl
siNrf2
H
ET-B
Relative mRNA expression
siControl
siNrf2
OA-NO2
I
BQ-788 + OA-NO2
ET-1 (pg/ml)
r2=0.8072
p<0.0001
Relative ET-R mRNA expression
J
BQ-788 + AdNrf2
ET-1 (pg/ml)
r2=0.3522
p=0.0022
Relative ET-R mRNA expression
K
BQ-788 + siNrf2
ET-1 (pg/ml)
r2=0.8071
p<0.0001
Relative ET-R mRNA expression
L
siNrf2 + OA-NO2
ET-1 (pg/ml)
r2=0.7666
p=0.0002
Relative ET-R mRNA expression

Figure 6