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ACTIVATION OF NRF2 COORDINATES DDAH/PPAR-γ/eNOS PATHWAYS THAT ENHANCE NITRIC OXIDE GENERATION IN HUMAN GLOMERULAR ENDOTHELIAL CELLS

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Abstract

Dimethylarginine dimethylaminohydrolase (DDAH) degrades (ADMA) which inhibits nitric oxide synthase (NOS). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcriptional factor that binds to antioxidant response elements (ARE) and transcribes many antioxidant genes. Since the promoters of the human DDAH-1 and -2, eNOS and PPAR- γ genes contain 2–3 putative AREs, we hypothesized that they were regulated by Nrf2/ARE. Incubation of human renal glomerular endothelial cells (HRGECs) with the Nrf2 activator tert-butylhydroquinone (tBHQ) (20 $\mu mol \cdot l^{-1}$) significantly ($P<0.05$) increased NO and activities of NOS and DDAH and decreased ADMA. It upregulated genes for hemoxygenase -1, eNOS, DDAH-1 and -2 and PPAR-γ and partitioned Nrf2 into the nucleus. Knockdown of Nrf2 abolished these effects. Nrf2 bound to one ARE on the DDAH-1 and -2 and PPAR-γ promoters but not to the eNOS promoter. An increased eNOS and phosphorylated eNOS (P-eNOSser-1177) expression with tBHQ was prevented by knockdown of PPAR-γ. Expression of Nrf2 was reduced by knockdown of PPAR-γ whereas PPAR-γ was reduced by knockdown of Nrf2, thereby demonstrating 2-way positive interactions. Thus, Nrf2 transcribes the HO-1 and other genes to reduce reactive oxygen species, and DDAH-1 and -2 to reduce ADMA and PPAR-γ to increase eNOS and its phosphorylation and activity thereby coordinating three pathways that enhance endothelial NO generation.

Keywords

asymmetrical dimethylarginine; endothelial dysfunction; tert-butylhydroquinone; antioxidant response element; cytoprotective genes

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that transcribes many antioxidant genes through a *cis*–acting antioxidant response element (ARE). Cytoplamic Nrf2 is bound to its inhibitor Kelch-like ECH-associated protein

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(Keap)-1. Specific cystein residues on Keap 1 can be oxidized during oxidative stress causing a conformational change that prevents ubiquitination and releases newly synthesized Nrf2 [1]. Other stimuli which can activate the pathway independent of oxidative stress [2], include bardoxolone methyl, naturally occurring substances such as rosveritrol or sulforaphane [3] or the small molecular weight substance *tert*-butylhydroxyquinone (tBHQ). tBHQ dissociates Nrf2-Keap1, stabilizes Nrf2 and phosphorylates it at Serine-40 Nrf2-P^{S40} via phosphatidylinositol 3-kinase (PI3K)/Akt [4, 5]. After dissassociation from Keap-1, Nrf2 and translocates it into the nucleus [6] where it dimerises with other transcription factors, notably mafmusculoaponeurotic fibrosarcoma oncogene homolog [7]. Specific binding to AREs initiates transcription of a plethora of cytoprotective genes including NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase-1 (GST-1), peroxisome proliferator activated receptor gamma (PPAR-γ), copper/zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2)[8].

Nitric oxide (NO) produced by eNOS in the vascular endothelium reduces vascular tone, mediates endothelium dependent relaxation and protects glomeruli from sclerosis and damage [9, 10]. eNOS activity is tightly regulated by expression, phosphorylation and availability of cofactors, and is inhibited by asymmetric dimethylarginine (ADMA) [11, 12] . Reactive oxygen species (ROS) enhance NO degredation and uncouple eNOS to redirect the enzyme to generate ROS [13]. ADMA is metabolized by two isoforms of dimethylarginine dimethylaminohydrolase (DDAH) to inactive products [11].

eNOS in the endothelium of the renal microvessels and glomeruli preserves glomerular function [14]. However, the effects of Nrf2 in human renal glomerular endothelial cells (HRGECs) has not been studied. tBHQ was selected as a potent activator of Nrf2 [15]. Since we detected 2 or 3 putative AREs in promoter regions of the human genes for eNOS, DDAH-1 and-2 and PPAR-γ, we tested the hypothesis that activation of Nrf2 by tBHQ regulated DDAH/ eNOS/ PPAR-γ transcription and thereby the metabolism of ADMA and the generation of NO. Primary cultures of HRGECs were selected since recent studies have shown that activation of Nrf2 with bardoxolene methyl increases the glomerular filtration rate in patients with diabetic nephropathy [16]. Moreover, single nucleotide polymorphisms of eNOS increase the risk of advanced diabetic nephropathy in type I diabetes mellitus [17]. Since ROS, endothelial dysfunction and ADMA predict cardiovascular disease (CVD) and progression of chronic kidney disease (CKD), interventions that may prevent or reverse these are attractive novel therapeutic targets [18–20].

Methods

Cell culture and proliferation of human renal glomerular endothelial cells (HRGECs)

Primary cultures were purchased from ScienCell Research Laboratories (Carlsbad, CA) and studied from passage 2 to 5 where eNOS protein expression was maintained (Supplement Figure S1). HRGEC proliferation was assessed from mitochondrial succinate-tetrazolium reductase using premixed WST-1 cell proliferation reagent (Clontech Lab.Mountain View, CA). (See Data Supplement).

Knockdown of Nrf2

HRGECS were transiently transfected with Nrf2 specific siRNA or control, non- targeted, siRNA (Santa Cruz Biotechnology,Inc. Santa cruz, CA).(See Data Supplement).

RNA isolation and real time quantitative RT-PCR

Real-time quantitative PCR was as described [21] (see Data Supplement).

Western blotting for protein in whole cell lysate or cell fractions

Immunoblotting assay was performed as described [21]. Nuclear and cytoplasmic fractions were prepared using the Cell Fraction System (BioVision Inc., Milpitas, CA) (See Data Supplement).

Medium nitrite, cellular NO and NOS and DDAH activities

Nitrite was measured using Griess reagent and NO generation from 4-amino-5-methylamino- 2', 7'-difluorofluorescein diacetate (DAF-FM DA) fluorescence. NOS activity was measured from conversion of [^{3}H] arginine to [^{3}H] citrulline and DDAH activity from conversion of [^{14}C]-ADMA to [^{14}C]-citrulline as described [21](see Data Supplement).

Medium ADMA

Medium ADMA was determined by capillary zone electrophoresis (see Data Supplement).

Chromatin immunoprecipitation assay (ChIP)

HRGECs were studied using EpiTect ChIP One Day Kit (SABiosciences, Frederick, MD) (see Data Supplement) after 24 hour incubation with 20μmol·l^{-1} tBHQ or vehicle.

Statistical analysis

Results are expressed as mean ± SEM with n=number of separate experiments. Each study was conducted in triplicate on 3 or more separate frozen vials of cells purchased at different times. Differences between experimental groups were compared by 2x2 ANOVA with interaction. Where appropriate, post-hoc testing was by Bonferroni t test. $P < 0.05$ was considered significant.

Results

Incubation of HRGECs for 24 hours with tBHQ enhanced cell proliferation maximally at 20 μmol·l^{-1} which was therefore selected for study (Figure 1 A). Knockdown of Nrf2 reduced basal and tBHQ-stimulated proliferation (figure 1B).

tBHQ (10–20 μmol·l^{-1}) increased ($P<0.01$) medium nitrite and intracellular NO by circa >60% which were prevented by knockdown of Nrf2 (Figure 2). Effects were maximal at 24hr (Supplemental Figure S2) which was selected for study. Incubation of HRGECs with tBHQ increased activity of NOS by 58±4% ($P <0.005$, figure 3A) and DDAH by 47±9% ($P < 0.01$, figure 3B) whereas knockdown of Nrf2 reduced basal and tBHQ-stimulated DDAH

activity and almost halved medium ADMA from 2.3±0.16 to 1.2 ± 0.02 μmol·l^{-1} (figure 3 C, $P < 0.001$).

As anticipated, tBHQ (20 μmol·l^{-1}) did not affect mRNA expression for Nrf2 [4, 5] but upregulated eNOS by 79 ± 9%, DDAH-1 by 129 ± 10% and DDAH-2 by 119 ± 25% (Supplemental Figure S3; all $P < 0.005$). All effects were prevented by knockdown of Nrf2. tBHQ did not change protein expression for Nrf2 but, similar to mRNA, increased eNOS protein by 43± 17%, DDAH-1 by 62 ± 21% and for DDAH-2 by 48 ± 18% (Figure 4; all $P < 0.05$). Nrf2 reduced basal protein expression of Nrf2 by 55%, and reduced basal expression of eNOS, DDAH-1 and -2 (figure 5). Figures 4 and 5 report Nrf2 immunoblots at 100 kDa corresponding to the poly-ubiquitanted form but another band was deleted at 58 kDa. Analysis of both yielded the same conclusions. Variable molecular weights for Nrf2 have been reported [22–24].

tBHQ stabilizes Nrf2 and translocates it to the nucleus. Cells treated for 24hr with tBHQ (20 μmol·l^{-1}) had increased Nrf2 protein in the nucleus, but not the cytoplasm (figure 6A). Lamin B-1 was the nuclear marker and LDH the cytoplasmic marker. There are 3, 3, 2 putative AREs on the promoters of eNOS, DDAH-1 and -2 located upstream (−1/−3000bp) of individual transcriptional start sites (TGACnnnGC or GCTGAGnnn) (See supplemental table S1) and functional AREs on the promoter of PPAR-γ [25]. ChiP assays from primary HRGECs (See supplemental table S2) demonstrated protein/DNA complexes that were immunoprecipitated by Nrf2 antibody and enriched by incubation with tBHQ covering ARE-2 for DDAH-1 (−1016/−1008) and ARE2 for DDAH-2 (−1152/−1143) but no nuclear binding to the eNOS promoter (figure 6B).

Since PPAR-γ increases eNOS expression and phosphorylation [26] and Nrf2 binds to AREs on the PPAR-γ promoter [25], we considered that PPAR-γ might mediate the effects of tBHQ on eNOS. Incubation of HRGECs for 24 hours with tBHQ (20 and 40 μmol·l^{-1}) increased PPAR-γ mRNA by 202 ± 12% (Figure 7A) with parallel increases in the protein expression for PPAR-γ and phosphorylated eNOS at amino acid residue Ser 1177(P-eNOS$^{ser\text{-}1177}$,Fig 7B). tBHQ significantly increased nuclear protein accumulation for Nrf2, Nrf2-p$^{s\ 40}$ (phosphorylated at Ser-40 of Nrf2) [4, 5] and the ratio of Nrf2-p$^{s\ 40}$ to Nrf2 (Supplemental Figure S4). Knockdown of Nrf2 reduced basal and tBHQ- stimulated mRNA for PPAR-γ (Supplemental Figure S5). Knockdown of PPAR-γ reduced basal levels of Nrf2, and reduced tBHQ-induced increases in gene expression for eNOS, DDAH-1 and DDAH-2 (Supplemental Figure S6) and led to parallel reductions in eNOS and p-eNOS$^{\mathbf{ser}\text{-}1177}$. However, the increase in expression of DDAH-1 or -2 persisted after PPAR-γ knockdown (Supplemental Figure S7). Finally, tBHQ greatly increased the gene expression for HO-1 and NQO1 (Supplemental Figure S8). These pathways are summarized in Figure 8.

Discussion

Activation of Nrf2 transcriptionally upregulates many oxidant defense pathways [8]. We confirmed robust upregulation by tBHQ of the mRNA expression for HO-1 and NQO-1.

The main new findings are that activation of AREs in HRGECs by tBHQ promotes cell proliferation, NOS activity and NO generation, increases DDAH activity, reduces ADMA and increases the expression of eNOS, DDAH-1 and -2 and PPAR-γ mRNAs and proteins. These effects were prevented by knockdown of Nrf2. While a ChiP assay identified one ARE site that bound Nrf2 and was enriched by incubation with tBHQ in both DDAH-1 and DDAH-2 gene promoters and PPAR-γ contains another [25], no binding site was detected on the eNOS promoter. Rather, the increased expression of eNOS protein with tBHQ was related to increased PPAR-γ expression since it was prevented by knockdown of PPAR- γ. Furthermore, tBHQ increased eNOS phosphorylation at serine 1177 which is a post-translational activation pathway. Thus, Nrf-2 activation may improve endothelial function by three distinct pathways (figure 8). First, it promotes gene expression for antioxidant molecules that counter the effects of ROS to impair endothelial function and uncouple eNOS. Second, it interacts with specific AREs on the DDAH-1 and -2 genes whose products enhance the degradation of ADMA which thereby lessen inhibition of eNOS. Third, tBHQ interacts with specific AREs on the PPAR-γ genes to enhance eNOS expression, phosphorylation and activity.

DDAH-2 metabolizes ADMA in blood vessels and maintains endothelial function whereas DDAH-1 regulates plasma levels of ADMA [11, 27] that predict cardiovascular events and death in high risk groups and the rate of loss of function in CKD [11, 18, 28]. The transcriptional regulation of DDAH expression is not well understood. Our study is one of few that have defined a pathway that can decrease ADMA [23, 29] and increase PPAR-γ and eNOS expressions and activity [25].

PPAR-γ agonists upregulate Nrf2 [25, 30]. Interestingly, we found that activation of Nrf2 by tBHQ increased the mRNA and protein expression for PPAR-γ whereas these were reduced by knockdown of Nrf2. Moreover, knockdown of PPAR-γ reduced the mRNA expression for Nrf2. This indicates a tight, positive, two-way reinforcing transcriptional interaction between PPAR-γ and Nrf2 that may improve endothelial function [31].

We acknowledge some limitations. First, we did not study the time course of Nrf2 nuclear binding after tBHQ incubation. Rather, we selected 24 hours for all studies since this was maximal for eNOS activation. Second, although we demonstrated specific nuclear binding of Nrf2 to the ARE-2 of DDAH-1 and -2, further studies of promoter transcriptional activity and ARE site mutation are required to definitely confirm its role in DDAH-1 and -2 gene transcription.

In conclusion, the established antioxidant actions of Nrf2 were complemented by upregulation of NO generation by increased NOS activity and ADMA metabolism by increased DDAH activity (figure 8). Thus Nrf2 can provide a coordinated regulation of the ROS/DDAH/PPAR-γ /eNOS pathways to protect endothelial function.

Perspectives

Increased ROS and ADMA have been implicated in early cardiovascular and renal disease [11, 32, 33]. The Nrf2 activator bardoxolone methyl given to patients with type 2 diabetes and CKD increased their eGFR by about 33% [16, 34], consistent with increased glomerular

endothelial NO generation. However, this potentially beneficial effect was offset by an unexplained increase in congestive heart failure [16]. Nevertheless, NO has multiple beneficial effects in blood vessels, the cardiovascular system, and the kidneys. NO generation is determined by NOS activity which in turn is increased by NOS expression that is promoted by DDAH [27]. Phosphorylation of eNOS at specific sites also increases NOS activity by ADMA and by uncoupling of the enzyme by ROS [13]. Remarkably, Nrf2 activation can promote NO activity by favorably influencing all of these pathways. Our study is the first to identify a strategy with a single drug (tBHQ) that not only reduces ROS, but enhances NO generation and PPAR-γ and reduces ADMA. Accordingly, drugs that activate Nrf2 could have widespread beneficial effects on the cardiovascular and renal systems, although the experience with bardoxolone methyl provides a cautionary note.

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Novelty and Significance

What is New?

- Activation of Nrf2 by tBHQ increases NO and PPAR-γ and decreases ADMA in human glomerular endothelial cells.
- tBHQ increases nuclear binding of Nrf2 that increases expression of DDAH-1 and 2 and PPAR-γ .
- eNOS and its phosphorlation with tBHQ are mediated by PPAR-γ transcription.
- There is one functional ARE site on DDAH-1and-2 promoters and one on PPAR- γ but none on eNOS.

What Is Relevant?

- Activation of Nrf2 promotes NO activity by multiple pathways (reduced ROS, increased eNOS transcription and phosphorylation and increased metabolism of ADMA)
- This provides a potential unique pathway to garner the many cardiovascular and renal benefits of enhanced NO

Summary

- An activated Nrf2 may promote endothelial function by a coordinated effect to limit oxidative stress and enhance the DDAH/ PPAR-γ /eNOS pathways.

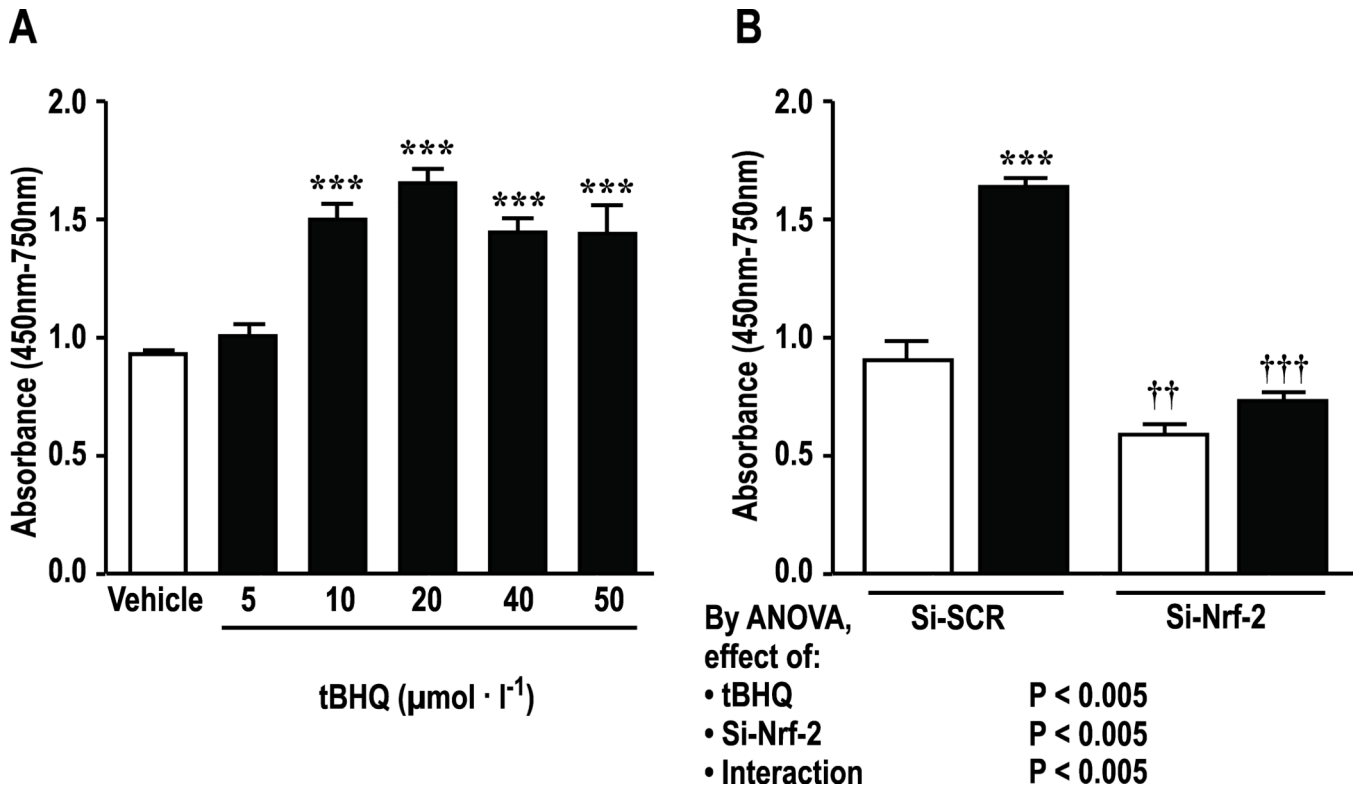

Figure 1.

Activation of Nrf-2 by tBHQ enhances proliferation of HRGECs. A, dose response for incubation with tBHQ over 24hr. B, effect of si-RNA to Nrf-2, compared to scrambled si-RNA. Si-SCR, scrambled si-RNA; Si-Nrf-2, Nrf-2-siRNA; open boxes, vehicle, solid boxes; tBHQ (20 µmol·l^{-1} in Panel B for 24 hr). Mean± SEM values (n=6 per group). Compared to vehicle; ***P < 0.005. Compared to si-SCR: ††, P<0.01; †††, P<0.005

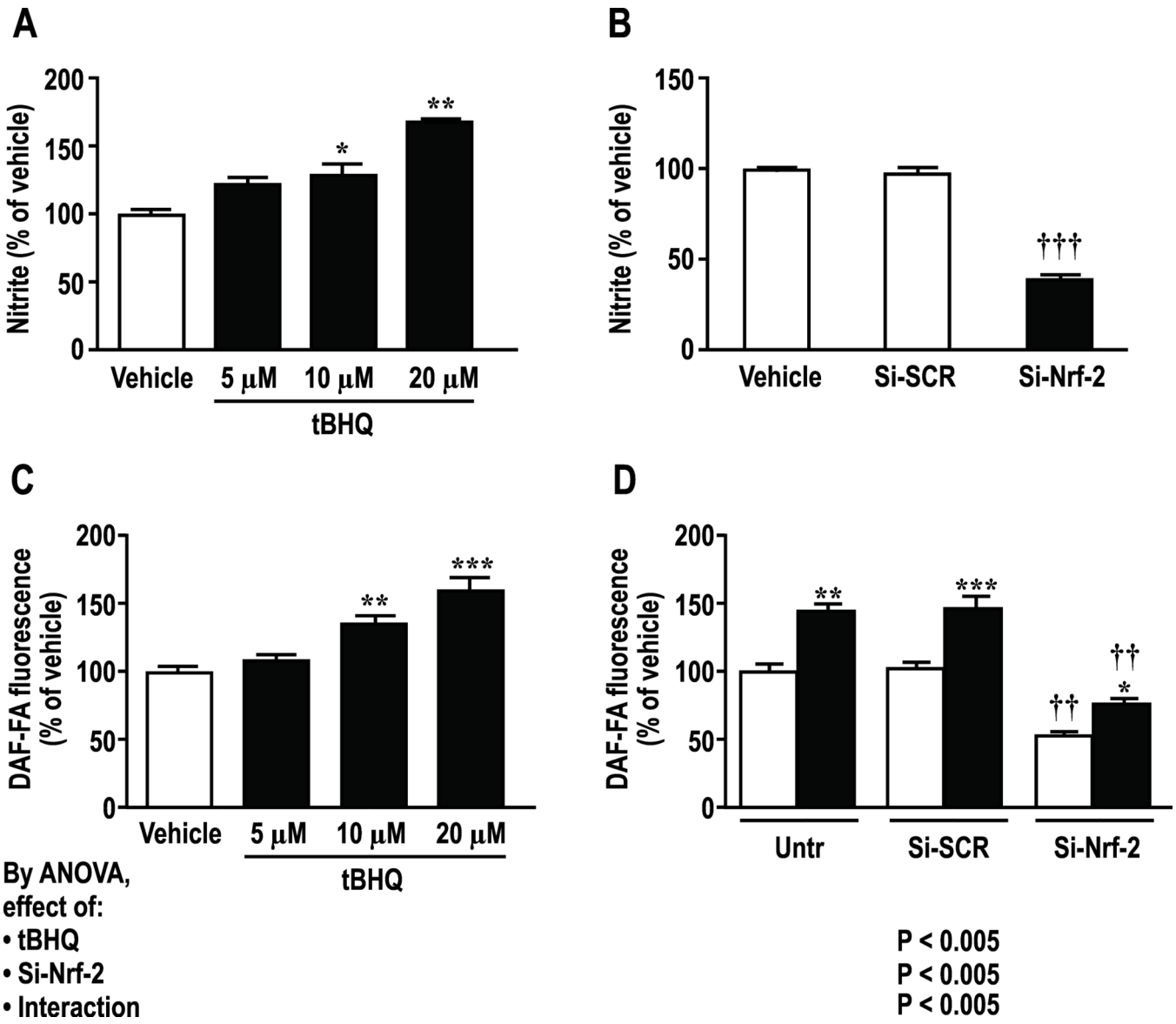

Figure 2.

Effects of tBHQ or si-RNA to Nrf-2 vs scrambled siRNA medium nitrite and intracellular NO activity over 24 hours. A and C, dose response; B and D effect of si-RNA to Nrf-2 vs. scrambled si-RNA in HRGECs. Si-SCR, scrambled si-RNA; Si-Nrf-2, Nrf-2-siRNA; open boxes, vehicle, solid boxes, tBHQ (20 μmol·l^{-1} in Panels B and D for 24hr). Mean ± SEM (n=6 per group). Compared to vehicle: *P < 0.05; **P < 0.01 and ***P < 0.005. Compared to si-SCR: ††, P<0.01; †††, P<0.005

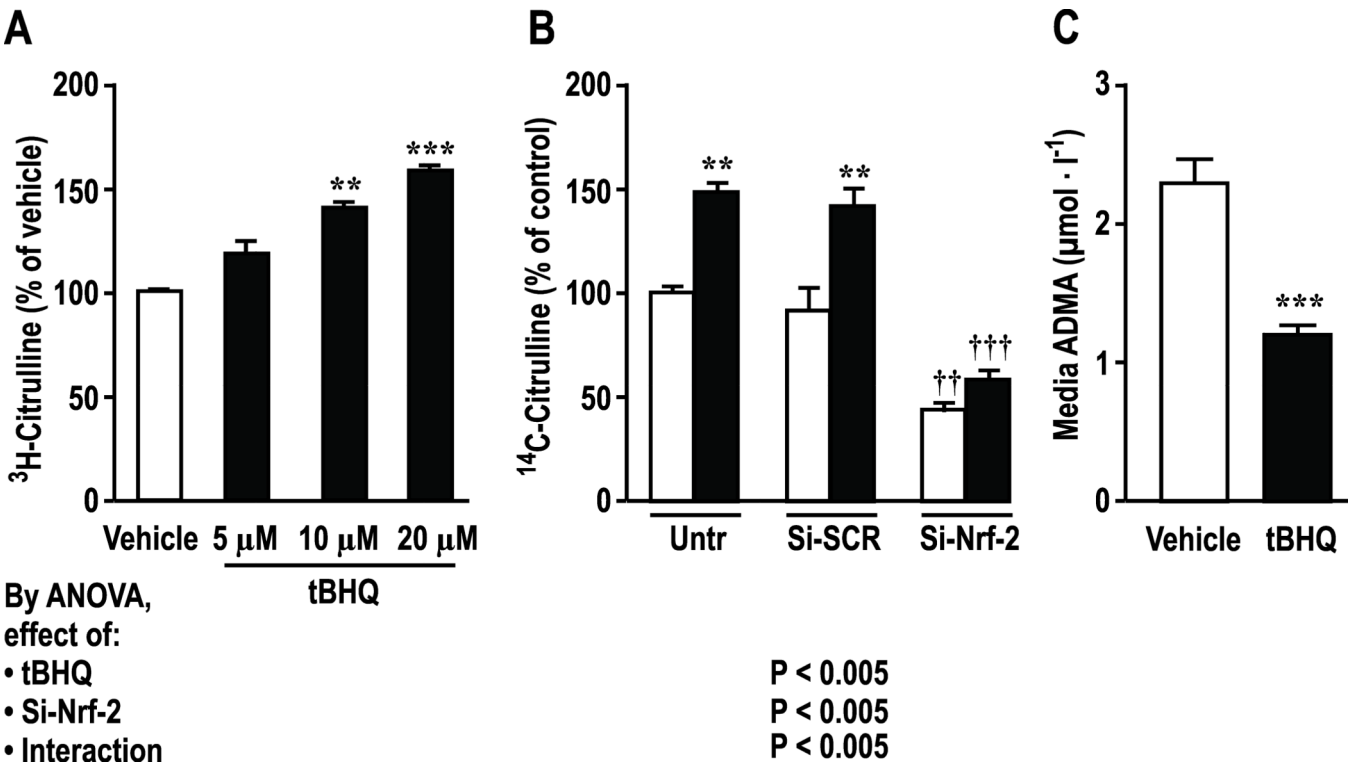

Figure 3.

Effects of tBHQ and si-RNA to Nrf-2 vs scrambeled siRNA on NOS activity (A), DDAH activity (B), and ADMA levels (C) in supernatants of HRGECs. Untr, untransfected control; Si-SCR, scrambled si-RNA; Si-Nrf-2, Nrf-2-siRNA; open boxes, vehicle, solid boxes, tBHQ (20 µmol·l^{-1} in Panels B and C). Mean ± SEM (n=6 per group). Compared to vehicle: **P < 0.01 and ***P < 0.005. Compared to si-SCR: ††, P<0.01; †††, P<0.005

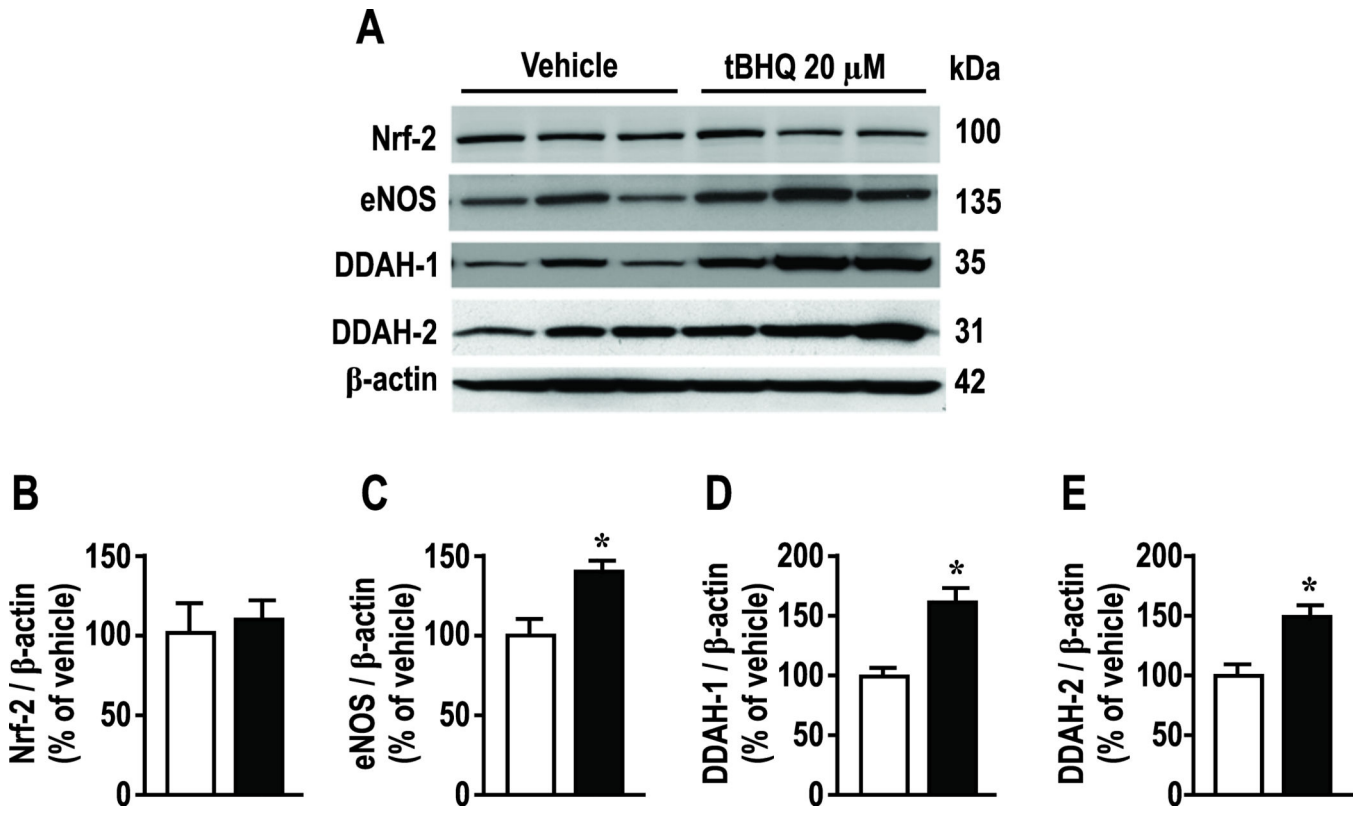

Figure 4.

Effects of tBHQ on protein expressions for Nrf-2, eNOS, DDAH-1 and DDAH-2 in HRGECs. Open boxes, vehicle, solid boxes, tBHQ (20 μmol·l^{-1} for 24hr). Mean ± SEM (n=6 per group). Compared to vehicle: *, P<0.05.

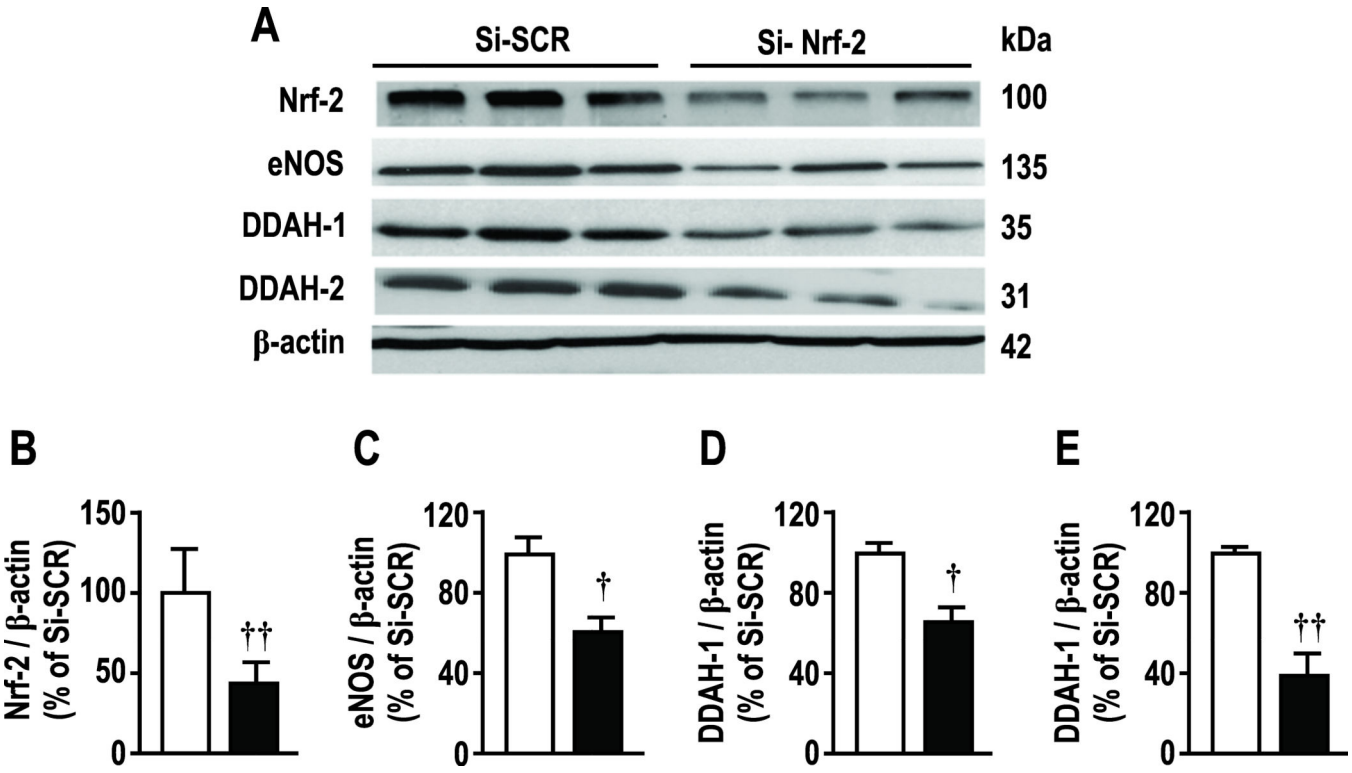

Figure 5.

Effects of transient transfection of Nrf-2 specific siRNA, compared to scrambled siRNA, on protein expressions of Nrf-2, eNOS, DDAH-1 and DDAH-2 in HRGECs. Si-SCR, scrambled si-RNA; Si-Nrf-2, Nrf-2-siRNA. Mean ± SEM (n=6 per group). Compared to si-SCR: *, P<0.05, **, P<0.01.

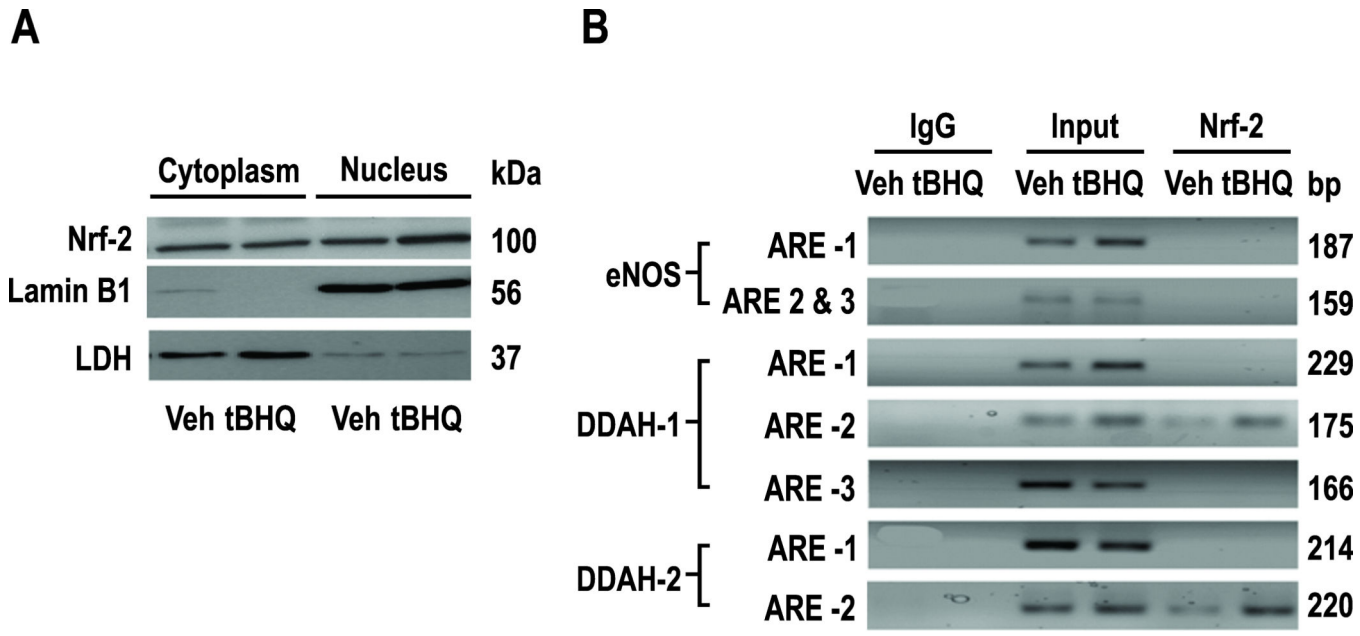

Figure 6.

Effects of tBHQ on translocation of Nrf-2 to the nucleus in HRGECs. Panel A, cytoplasmic and nuclear expressions of Nrf-2 and enhancement of nuclear expression by tBHQ (20 μmol·l^{-1}). Panel B, chip assays showing Nrf-2 binding to ARE-2 on promoters for DDAH-1 and -2 and enhancement by tBHQ (20 μmol·l^{-1}), but no nuclear binding, to AREs on eNOS.

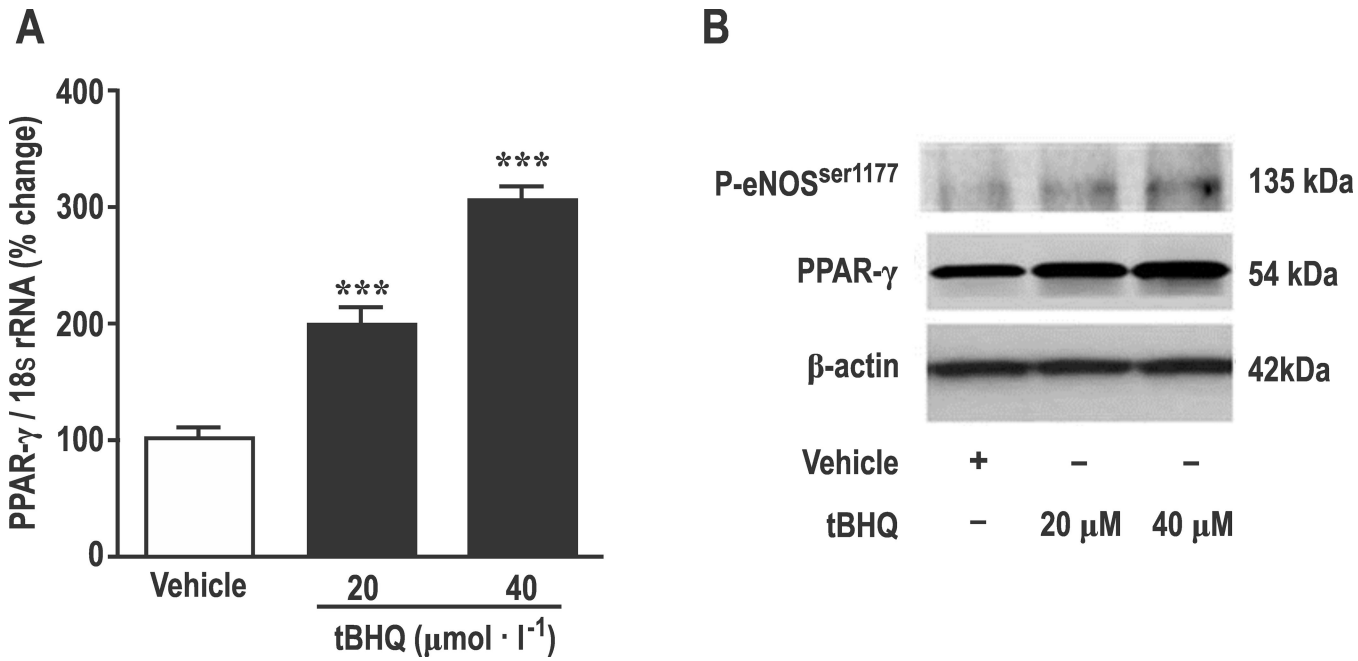

Figure 7.

Effects of tBHQ on mRNA expressions for PPAR-γ (Panel A), protein expression for p-eNOS^{ser1177}, and PPAR-γ (Panel B) in HRGECs. Open boxes, vehicle, closed boxes, tBHQ. Compared to vehicle in A: ***, P<0.005. Blot band images shown in B are representatives from 3 independent experiments.

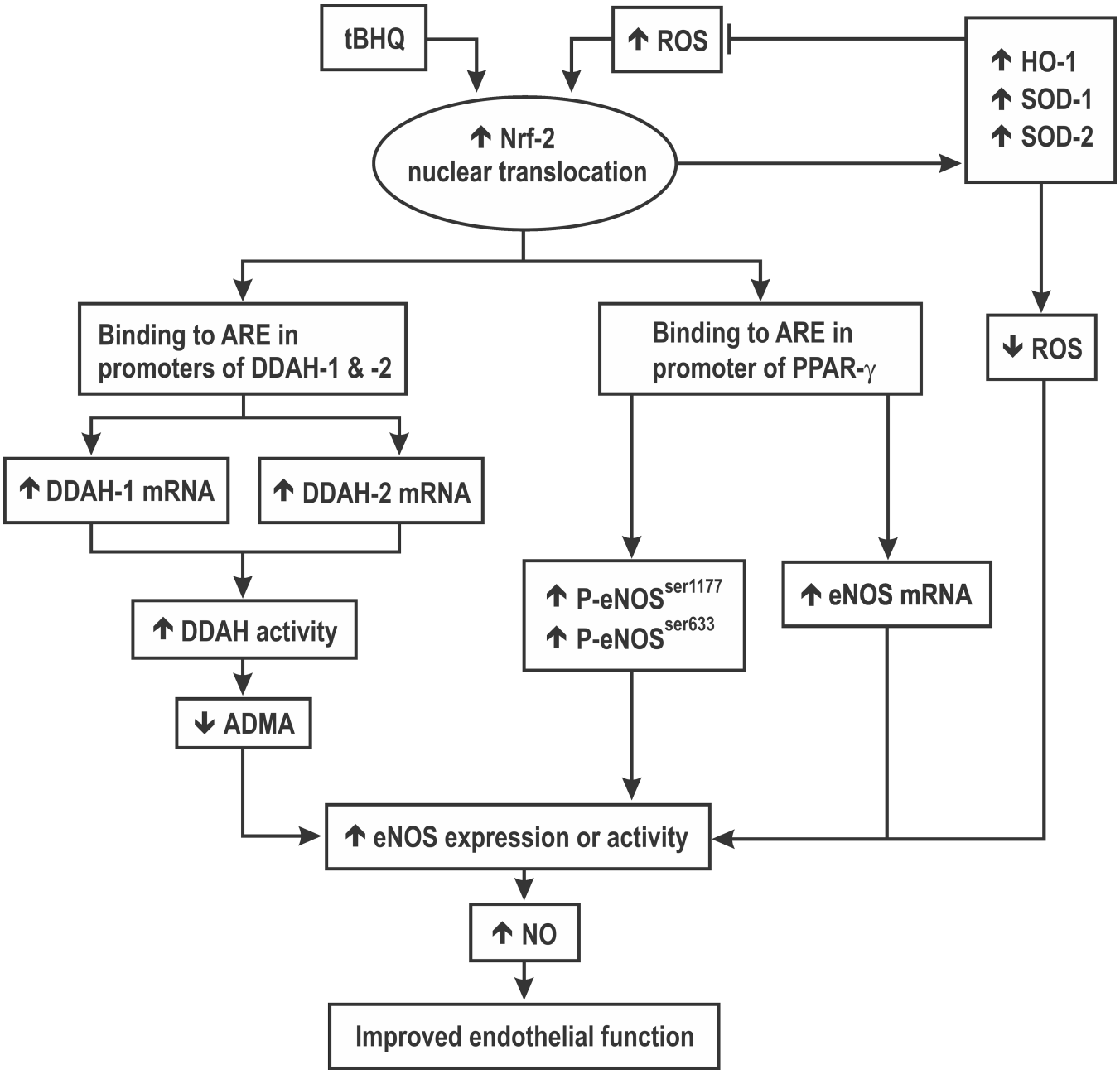

Figure 8.

Schematic hypothesis for roles of Nrf-2 in preserving endothelial function during activation by tBHQ or reactive oxygen species.