Interplay between Vascular Endothelial Growth Factor (VEGF) and the Nuclear factor erythroid 2-related factor-2 (Nrf2): implications for preeclampsia*

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*Running title: VEGF activates Nrf2

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Keywords: Nrf2, VEGF, HO-1, carbon monoxide, preeclampsia, oxidative stress.

Background: Several studies have suggested that decreasing VEGF levels might result in placental oxidative stress in preeclampsia.

Results: VEGF activates Nrf2 in an ERK1/2-dependent manner protecting against oxidative stress and, in turn, up-regulates VEGF expression.

Conclusions: Reduced VEGF bioavailability may lead to aggregation of oxidative stress and end up in preeclampsia.

Significance: Nrf2 activation might be considered as an adjunct therapeutic strategy to combat preeclampsia.

SUMMARY
Several recently published studies have suggested that decreasing Vascular Endothelial Growth Factor (VEGF) levels result in placental oxidative stress in preeclampsia, although the question how decreased VEGF concentrations increases oxidative stress still remains unanswered. Here we showed that VEGF activated the Nuclear factor erythroid 2-related factor-2 (Nrf2), the main regulating factor of the intracellular redox balance, in the cytotrophic cell line BeWo. This, in turn, activated the production of antioxidative enzymes, thioredoxin, thioredoxin reductase and heme oxygenase-1 (HO-1), that showed a decrease in their expression in the placenta of preeclamptic women. Nevertheless, this activation occurred without oxidative stress stimulus.

As a consequence, the activation of Nrf2 protected BeWo cells against H₂O₂/Fe²⁺-induced oxidative damage. We further showed that VEGF up-regulated the expression of itself. A positive feedback loop was described in which VEGF activated Nrf2 in an ERK1/2-dependent manner; the upregulation of HO-1 expression by Nrf2 augmented the production of carbon monoxide, which in turn up-regulated VEGF-expression. In conclusion, VEGF induces the Nrf2 pathway to protect against oxidative stress and, via a positive feedback loop, to elevate the expression of VEGF. Therefore, decreased VEGF bioavailability during preeclampsia may result in a higher vulnerability to placental oxidative cell damage and to a further reduction of VEGF bioavailability; a vicious circle that may end up in preeclampsia.
INTRODUCTION
Preeclampsia (PE) is unique to human pregnancy and is characterized by proteinuric hypertension in up to 3-5% of all pregnancies (1). In developing countries where access to health care is limited, preeclampsia is a leading cause of maternal mortality, with estimates of >60,000 maternal deaths per year (2). Despite intensive research, the etiology of preeclampsia still remains unclear. Pathogenic factors affecting both fetus and mother are currently under intensive investigation: in the fetus, the principal pathology appears to be placental oxidative stress, resulting from insufficient uteroplacental blood supply (3), whereas the underlying maternal pathology is a severe systemic inflammatory response involving leukocyte and endothelial cell activation (4-6).

Vascular Endothelial Growth Factor (VEGF) expression takes place in the villous trophoblast (7-9). Upon gene activation, the resulting VEGF-receptor initiates a cascade of cellular protein phosphorylation reactions by several protein kinases and leads to a variety of cellular responses (10-12). One family of these downstream kinases is the mitogen-activated protein kinases (MAPK) p42 and p44, also referred to as ERK1/2. ERK1/2 is phosphorylated and activated by MAPK kinase [MAPK kinase (MAPKK) or MEK] in the cytosol, translocates to the nucleus, and subsequently stimulates transcription of early response genes regulating cell proliferation and survival (10-12).

Maynard et al. reported that the soluble VEGF receptor, soluble fms-like tyrosine kinase-1 (sFlt-1 or sVEGFR-1), is involved in the pathophysiology of preeclampsia (13). More recently, it was reported that elevated maternal sFlt-1 and decreased VEGF concentrations result in increased oxidative stress which contributes to vascular dysfunction during pregnancy. Although the question is raised about how decreased VEGF concentrations increases oxidative stress, still remains unanswered (14).

Oxidative stress in syncytiotrophoblasts of women with preeclampsia is well documented (3,6). These cells are especially sensitive to oxidative stress partly because of their location in the outermost layer of placental villi where they are exposed to high oxygen concentrations, and partly because they contain surprisingly low concentrations of antioxidant enzymes (15,16).

A battery of genes encoding antioxidant enzymes is orchestrated upon exposure to reactive oxygen species. This coordinated response is regulated via the Antioxidant Response Element (ARE) contained within the regulatory regions of so-called “safeguard” genes such as NAD(P)H, quinone oxidoreductase-1 (NQO1), thioredoxin reductase (TXNRD), glutathione peroxidase (GPX), and heme oxygenase-1 (HO-1) (17,18). Activation of the nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), as a consequence of oxidative stress, initiates and enhances transcription of these “safeguard” genes, thus protecting cells against oxidative stress as well as a wide range of other toxins (19-23).

Mann et al. were the first to discuss a link between Nrf2, vascular homeostasis and preeclampsia (24). Recently, Wruck et al. have shown first experimental data that Nrf2 is exclusively active within cytotrophoblasts of preeclamptic placenta, strongly suggesting that these cells suffer from oxidative stress caused by reactive oxygen species (25). In the current paper it is hypothesized that oxidative stress during preeclampsia results in increased expression and transfer of antioxidant enzymes from cytotrophoblasts into syncytiotrophoblasts via enhanced syncytial fusion, thereby increasing cytotrophoblast proliferation and syncytial knot formation (necrotic and aponectotic subcellular syncytial fragments).

Thus the principal aim of this paper is to investigate whether VEGF activates Nrf2 which counters oxidation stress. The second objective is to study whether the activation of Nrf2 which leads to an increase in cellular carbon monoxide, raises VEGF levels.

MATERIAL & METHODS
Cell culture and stimulation- Human choriocarcinoma cell line BeWo were obtained from ATCC. The cells were cultured in Ham’s F-12 (PAA Laboratories GmbH, Austria) with 10 % fetal bovine serum FBS (GIBCO®, Invitrogen Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO®, Invitrogen Life Technologies) and incubated at 37°C. The cells were seeded into petri dishes (Ø 10 cm) (2 x 10⁶ cells/dish), 6-well plates (5 x 10⁵ cells/well) or 96-well plates
(10 x 10^4 cells/well) for subsequent culture. The cells were then cultured in 20% O_2 for 24 h and then incubated overnight with 50 µM vitamin C (Sigma-Aldrich Co, Germany) to attenuate a pre-stimulation of Nrf2.

These cells were subsequently stimulated with 10 ng/ml human VEGF_165 (R&D Systems, Inc., Germany) or were left unstimulated as the control. VEGF_165, an isoform of VEGF-A, was used in this study because it was supposed be one of the most potent angiogenic isoforms (26). For positive control of Nrf2 activation, some cells were treated with 1 µM sulforaphane (Sigma-Aldrich Co, Germany) (27,28).

Oxidative stress was induced with hydrogen peroxide (ROTH, Germany) in the presence of 10 µM iron (II) sulfate (Sigma-Aldrich Co, Germany) (29).

For the experiments with MAPK, p38, c-JNK and PI3K inhibitors, the cells were seeded into 6-well or 96-well plates. After reaching confluence, the cells were incubated with 50 µM vitamin C before they were exposed to kinase inhibitors. The applied inhibitor concentrations were as follows: MEK1 inhibitor PD98059 1-50 µM (Cell Signaling Technology, Inc, USA); the MEK1/2 inhibitor U0126 1-10 µM (Cell Signaling Technology, Inc., USA); p38 inhibitor SB203580 1-40 µM (Calbiochem, Germany), c-Jun N-terminal kinase inhibitor SP600125 1-50 µM and PI3K inhibitor Wortmannin 0.1-5 µM (Calbiochem, Germany). Diphenyliodonium chloride in concentrations of 1-20 µM was used as a specific inhibitor of NAD(P)H oxidase (Sigma-Aldrich Co, Germany). The cells were pretreated for 30 minutes with inhibitors before VEGF was added for additional 1 h prior to the assay.

To test the effect of carbon monoxide, the tricarbonyldichlororuthenium (II) dimer (CORM-2) (Sigma-Aldrich Co, Germany) was applied. The cells were incubated in Ham’s-F12 medium containing 10% FCS and the indicated concentration of CORM-2 for 24 hours. To scavenge CO, human hemoglobin (Hb) (Sigma-Aldrich Co, Germany) was first dissolved in phosphate-buffered saline (PBS) under an argon atmosphere and agitated at room temperature for 2 h prior to filter sterilization. Various doses of Hb were added to the Ham’s F-12 medium at the same time as the administration of VEGF_165. After the stimulation, the cells were lysed and VEGF protein concentrations were measured by ELISA Assay.

**Cell lysate preparation and immunoblot analysis** - Cells were lysed in a buffer containing 10 mM HEPES (PromoCell GmbH, Germany), 1.5 mM MgCl_2 (Sigma-Aldrich Co, Germany), 10 mM KCl, 0.5 mM DTT, 0.05% NP-40, at pH 7.9 and protease inhibitors, and the lysate were left on ice for 10 min. Cytosolic fractions were obtained as the supernatant after centrifugation at 950 x g for 10 min at 4 °C. The pellets were resuspended in nuclear extraction buffer containing 5 mM HEPES, 1.5 mM MgCl_2, 0.2 mM EDTA (GIBCO®, Invitrogen Life Technologies), 0.5 mM DTT, 26% glycerol and protease inhibitors at pH 7.9. Afterwards, 4.6 M NaCl was added followed by a 30-min incubation-step on ice. Nuclear proteins were found in the supernatant after a 20-minutes centrifugation at 24,000 x g at 4°C, (abcam protocol). The protein (6 µg) of the nuclear fractions and (15 µg) of the cytosolic fractions were subjected to 12.5% discontinuous sodium dodecyl sulphate polyacrylamide gels, separated by electrophoresis, and then electroblotted onto PVDF membranes (Millipore GmbH).

The immunoblot analysis was performed with specific antibodies and enhanced chemiluminescence-based detection kit (Millipore GmbH). Antibodies against Nrf2, HO-1, TRX, and TXNRD1, p38 were purchased from (Abcam, UK); β-actin, pERK, ERK were purchased from (Santa Cruz Biotechnology, Inc., Germany), and histone 3 (H3), AKt, pAKt (Ser 473), phospho-p38 were brought from (Cell Signaling Technology, Inc, USA).

The densities of the bands were measured via PCbas 2.0 software, and the ratio between the protein and the corresponding loading control (histone H3 and β-actin for Nrf2, β-actin for Trx, TXNRD1, HO-1, total ERK1/2, phospho-β-actin for pAKt) was calculated.

**The Dual Luciferase Assay- ARE of the rat NQO1- gene was cloned into the pGL3-promoter vector (Promega, Madison, Wis, USA) as described in (21). The pGL3-ARE vector was co-transfected with renilla luciferase plasmid phRL-TK (Promega, Madison, Wisconsin, USA) by using jetPRIME™ transfection reagent (Polyplus-transfection Inc, France) according to the manufacturer’s recommendations. Twenty-four hours after transfection the cells were seeded in a 96-well plate. The activities of both firefly and renilla luciferases were determined 48 h after
transfection with the dual luciferase reporter gene assay system (Promega) in the 96-well plate reader (GloMax®-96 Microplate Luminometer, Promega Corporation). The firefly luminescence signals were normalized to the corresponding renilla luciferase signals acting as internal control.

**Nrf2 silencing-** BeWo cells were transfected with 10 µg of control-shRNA and Nrf2-shRNA. The plasmids were part of the MISSION® shRNA assortment purchased from Sigma-Aldrich Co. (Germany). The aforementioned cells were transfected using jetPRIME™ transfection reagent according to the manufacturer’s instructions. After 24 h the medium was replaced with fresh Ham’s F-12 with 10% FBS; Puromycine (ROTH, Germany) was added 48 h after transfection. The cells were grown in the medium containing 1 µg/ml of puromycin to obtain a sufficient selection. Stable cell lines were established once all the cells in the negative control plate were killed. These stable cell lines were continuously grown in the medium containing 1 µg/ml puromycin. After about 10 passages, the gene expression levels of Nrf2 were measured by quantitative RT-PCR in order to check the knockdown from Nrf2 transcription in these cells.

**Reverse transcription and PCR-** Cells were harvested in peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, Germany) for extracting RNA according to the manufacturer’s protocol. Hereby RNA concentration was determined by photometric analysis with the NanoDrop1000 system (PEQLAB GmbH, Germany), then 1 µg of total RNA was digested with DNase I (Roche GmbH, Germany) and transcribed into cDNA by reverse transcription with RevertAid™ Reverse Transcriptase (Fermentas Life Sciences, Canada). Real-time PCR reactions were processed in triplicate using the ABI StepOnePlus™ apparatus (Applied Biosystems, USA) in a total volume of 20 µl containing 100 ng cDNA, gene specific primers and including SYBR Green I reagent (Applied Biosystems, USA). The gene expression was determined by normalizing the target gene Ct values to the expression of the housekeeping gene, which was 18S in this study (Eurofins MWG Operon, Germany). The following sequences for 18S primer (forward: 5’-ATGTGGTAGCCGTTTCTCAGGC-3’; reverse: 5’-ATGTCGCGCCAAAAGCTG-3’).

Annealing temperature: 61.5 °C were applied.

Nrf2 primer: (Eurofins MWG operon, Germany). (forward: 5’-TCCAGTCAGAAACCAGTGGAT-3’; reverse: 5’-AATGTCGTCCGAAAAAGCTG-3’).

Annealing temperature: 60.5 °C.

**Assay for intracellular redox- state** Intracellular redox state levels were measured using the fluorescent dye, H2-DCFH-DA (Invitrogen Life Technologies) as described in (30). Briefly, cells were washed once with phosphate-buffered saline and incubated in the same buffer containing 10 µg of DCFH-DA for 30 min at 37°C. Intracellular fluorescence was detected with Ex485/Em530 using Spectra Max Gemini EM (Molecular Devices, CA).

**Cell viability assay-** WST-1 assay- For the 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay, the media were supplemented with 10 µl/well (96 well plate) WST-1 (Roche, Germany). The spectrophotometric evaluation was performed after 1, 2 and 4 h. Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (Model680, Bio-Rad, Hercules, CA, USA). This reaction reflects the reductive capacity of the cells, which represents the viability of the cells which is expressed as a value of 1. This value 1 represents the reductive capacity of the untreated control.

**Cytotoxicity assay-** CytoTox-Glo™ Cytotoxicity assay (Promega Co.) was used to measure cytotoxicity. The cells were seeded in 96-well plates and starved overnight by adding FCS-free medium supplemented with vitamin C. The cells were treated with or without 10 ng/ml VEGF165 for 3 h, then all cells were incubated with 500 µM H2O2/10 µM Fe2+ for 6 h. The CytoTox-Glo™ Cytotoxicity Kit was used according to the manufacturer’s recommendations. The luminescence was measured in a 96-well plate reader (GloMax®-96 Microplate Luminometer; Promega Co.).

**ELISA Assay-** The cells were seeded in 6-well plates; after reaching confluence, the cells were treated with VEGF165 for 6 h. Subsequently, the cells were washed twice with phosphate-buffered saline, lysed or supplied with new medium (without FCS) for 24 h; the
levels of VEGF Protein in the supernatant or in the cellular lysate were assessed by a sandwich ELISA (R&D Systems Minneapolis, MN; USA) that detects all VEGF splice forms. Human recombinant VEGF165 was used for standard curve determination.

Statistical analysis. All measurements were performed at least in triplicates and the results were expressed as the mean ± SEM. Statistical analyses were performed using unpaired Student's t-test procedures for dual comparisons. Mean differences were considered to be significant when p < 0.05. All statistical graphs and analyses were created with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA USA).

RESULTS

VEGF165 increased Nrf2 activation and Nrf2 target gene expression. To investigate the efficacy of VEGF165 to activate the cis-acting element ARE, a dual luciferase reporter gene assay was performed with ARE of the NQO-1-gene. ARE-activation was determined in a dose-response assay in concentrations up to 10 ng/ml VEGF165 on BeWo cells for 6 h incubation. According to Fig 1 A, the relative ARE activity for VEGF165 (10 ng/ml) was approximately twice that of the control. This is also true for the positive control, 1 µM sulforaphane.

It was subsequently tested if this ARE activation is Nrf2-dependent. Therefore, a shRNA against mRNA coding for human Nrf2 was designed, whereby a BeWo cell line (BeWo-shNrf2) and a control BeWo cell line containing a scrambled control shRNA (BeWo-shCont) were produced and stable transfected in BeWo cells. Real-time RT-PCR was used to test the efficacy of the shRNA against Nrf2. Real-time RT-PCR analysis showed an Nrf2 knock-down of >90% in the BeWo-shNrf2 in both untreated and 1 µM sulforaphane treated cells. In contrast, sulforaphane treatment showed a significant activation of Nrf2 mRNA in BeWo-shCont (Fig. 1 B, upper graph).

The Nrf2-specific shRNA totally abolished the VEGF165- as well as the sulforaphane (positive control)-dependent ARE activation. These results demonstrate that Nrf2 is the transcription factor that activates ARE expression in response to VEGF165 (Fig. 1 B).

Nrf2 is a critical regulator of the intracellular antioxidants and phase II detoxification enzymes as an adoptive response to oxidative stress or pharmacological stimuli. To investigate whether VEGF165 induces Nrf2 activation, 10 ng/ml VEGF165 was administrated to the cells at various time points. As early as 1 h following the administration of VEGF165, Nrf2 accumulated in the nucleus of the BeWo cells and remained at an elevated level for at least 6 h (Fig. 1 C). Figure 1 E presents the mean of three independent experiments. Subsequently, the protein expression of three Nrf2 target genes, i.e., thioredoxin (Trx), thioredoxin reductase 1 (TXNRD1), and heme oxygenase-1 (HO-1), was elevated 3 h after the administration of VEGF165 (Fig. 1 D). The mean of three independent experiments is shown in Fig. 1 F.

Nrf2 activation by VEGF165 was ERK1/2 dependent. To address the role of MAPK-pathways in ARE gene regulation by VEGF165, the effects of various kinase inhibitors were examined. Hence, the activation of ERK1/2 was found to be a prerequisite for the activation of Nrf2 by VEGF165 since VEGF165-mediated Nrf2 activation is exclusively inhibited by the MEK1/2 inhibitor PD98059 (50 µM) as well as the MEK1/2 inhibitor U0126 (10 µM) did (Fig. 2 A). The c-Jun N-terminal kinase inhibitor SP600125 (1-50 µM), the inhibitor of p38 MAPK SB203580 (1-40 µM), Wortmannin (0.1-5 µM) an inhibitor of phosphoinositol-3-kinase (PI3K), did not affect ARE activation in VEGF165 stimulated BeWo cells (Fig. 2 A).

To confirm these results the effect of VEGF on both p38 and Akt activation were tested consequently. VEGF stimulation did not up-regulate p38 phosphorylation over time. In contrast, Phorbol myristate acetate (PMA) which, was used as positive control, induced a phosphorylation of p38 and this phosphorylation was blocked by 20 µM SB203580 (supplemental Fig. 1 A and B). On the other hand hydrogen peroxide was used as a positive control for Akt activation (31). VEGF stimulation did not up-regulate Akt phosphorylation over time. In contrast, hydrogen peroxide induced a phosphorylation of Akt and this phosphorylation was blocked by 1 µM Wortmannin (supplemental Fig. 1 C and D).

Next diphenyliodonium chloride (DPI) was used as a NAD(P)H oxidase inhibitor in order to elucidate the role of NAD(P)H oxidase and ROS generation in the signal transduction of VEGF. (Fig. 2 A). To test if stimulation with VEGF induces ROS production we monitored the levels ROS with the fluorescent dye, H2-
cells were treated with 10 ng/ml VEGF peptides contained in these residues. BeWo detected by antibodies against phosphorylated sequence, and the activated species could be ‘activation lip’ of the conserved core kinase of threonine and tyrosine residues located in the ERK1/2 was activated by dual phosphorylation.

Stress-induced Nrf2 activation revealed that VEGF was shown in (Fig. 2 C). The results showed clearly that NAD(P)H oxidase and ROS generation is not involved in the VEGF-induced Nrf2 activation.

To determine the role of ERK1/2 in VEGF mediated Nrf2 activation, the effect of VEGF on ERK1/2 activity was examined. ERK1/2 was activated by dual phosphorylation of threonine and tyrosine residues located in the ‘activation lip’ of the conserved core kinase sequence, and the activated species could be detected by antibodies against phosphorylated peptides contained in these residues. BeWo cells were treated with 10 ng/ml VEGF, and cell extracts were analyzed for phosphorylated and total ERK1/2 by Western blotting. The loading control via total ERK1/2 Western blot showed no significant differences (Fig. 2 B). The mean of three independent experiments is shown in (Fig. 2 C). The results showed clearly that VEGF activates ERK1/2 and that 50 µM PD98059 as well as 10 µM U0126 inhibit this ERK1/2 activation in comparison to the treated cells with VEGF, which was supposed to be the positive control.

Consequently, inhibition of ERK1/2 either by 50 µM PD98059 or by 10 µM U0126 significantly blocked the VEGF-induced nuclear accumulation of Nrf2 (Fig. 2 D), and the treated cells with VEGF without inhibitor was considered as the positive control. The mean of three independent experiments is shown in (Fig. 2 E).

Nrf2 activation by VEGF conferred cytoprotection against oxidative stress. The hypothesis that activation of Nrf2 could protect BeWo cells from lesions caused by oxidative stress via H2O2 treatment was tested. Therefore, BeWo cells were preincubated with 10 ng/ml VEGF for 3 h. Then the cells were treated with 500 µM H2O2 + 10 µM Fe2+. Cell viability was measured via the WST-1 assay, and cell death via CytoTox-GloTM Cytoxicity assay 6 h after H2O2 administration. These assays revealed that preincubation of BeWo cells with 10 ng/ml VEGF effectively protects the cells from H2O2-induced toxicity in both the WST-1 assay (Fig. 3 A) as well as in the CytoTox-GloTM Cytoxicity assay (Fig. 3 C).

A causal relationship between Nrf2 activation and cytoprotection mediated by VEGF was further investigated. As shown in (Fig. 3 A – D), VEGF could protect BeWo-shControl cells but not BeWo-shNrf2 cells from oxidative toxicity. After incubation with 500 µM H2O2 + 10 µM Fe2+, the cell viability of BeWo-shNrf2 cells declined by 50% (Fig. 3 B) versus the 20% decline which was found with BeWo-shControl cells (Fig. 3 A). Consistently, BeWo-shControl cells showed a significant 30% decrease in cell death after 500 µM H2O2 + Fe2+ treatment in the presence of VEGF-treatment (Fig 3 C), whereas VEGF-treatment in the BeWo-shNrf2 cells had no effect (Fig. 3 D).

**VEGF up-regulated VEGF via Nrf2 / HO-1 / CO.** To evaluate whether the treatment with VEGF has an effect on the production of VEGF protein itself in BeWo cells, both BeWo-shControl and BeWo-shNrf2 cells were treated with 10 ng/ml VEGF for 6 h. Then 24 h after the stimulation, both cell lines showed elevated levels of VEGF protein itself in the supernatants (Fig. 4 A, black bars). Nrf2-silencing reduced the VEGF-induced increase in VEGF itself by 30% in comparison with BeWo-shControl cells, which infers that partial upregulation of VEGF protein expression was attributed to at least one of Nrf2-mediated product (Fig. 4 A, gray bars). Next we tested if Nrf2 activators induce VEGF expression in our system. BeWo-shControl cells stimulated with 1µM Sulforaphane and 1 mM Bipyridyl (33) showed a twofold increase of VEGF expression compared to control. In contrast, BeWo-shNrf2 cells are not able to up regulate VEGF expression in response to Sulforaphane (Fig 4 B).

Due to carbon monoxide produced by exogenous sources or the degradation of heme by HO-1, the gene expression for VEGF protein is induced (34). Therefore the effect of CO on VEGF expression in BeWo cells was examined. CORM-2 treatment significantly increased VEGF protein levels in a low dose-dependent manner (Fig. 4 C, left Y-axis) in comparison with untreated cells. Since a higher dose of CORM-2 (50 µM) was toxic it did not further induce VEGF expression (Fig. 4 C, right Y-axis).

It was then examined whether CO is essential for VEGF-induced VEGF up-regulation. Cell treatment with the CO scavenger hemoglobin (Hb) (10 -100 µM) showed a dose-dependent inhibition of VEGF-induced VEGF expression (Fig. 4 D).
These results clearly indicate that VEGF-up-regulated VEGF expression depends on Nrf2 and HO-1.

DISCUSSION

Several recently published results have suggested that decreasing VEGF levels might result in placental oxidative stress in preeclampsia (13,35,36). Experimental animal studies have demonstrated that elevated maternal sFlt-1 and decreased VEGF concentrations result in increased oxidative stress (14). Although the occurring question how decreased VEGF concentrations increases oxidative stress still remains unanswered.

A recent study suggests a possible role of Nrf2 in mechanisms underlying preeclampsia. The study used genome-wide transcriptional profiling of preeclamptic and normal pregnancies and showed that Nrf2-mediated oxidative stress response was overrepresented in preeclampsia (37) Thus, these data strengthen our previous published results (25) and our hypothesis that Nrf2 has a critical role in the etiology and progression of preeclampsia.

Nrf2 plays a key role in the adaptive response to oxidative and electrophilic stress by maintaining the cellular self defense. This current study provides substantial experimental evidence that VEGF activates Nrf2 as well as up-regulates Nrf2-dependent gene products within the well established cytotrophic cell line BeWo (38) (Fig. 1).

Several studies have shown that protein kinases are involved in the activation of Nrf2 (39). Since it is also known that binding of VEGF to VEGF receptor-2 activates downstream effectors including PKC, Raf and ERK1/2, PI3K and FAK pathways. Therefore, it was examined whether kinases are involved in the signal transduction of VEGF leading to Nrf2 activation. Thus, various kinase inhibitors with regarding Nrf2 activation were tested. From all tested inhibitors, only the MEK1/2 inhibitor PD98059 and U0126 inhibited the Nrf2 activation. Consequently, ERK1/2 activation is a prerequisite for Nrf2 activation by VEGF. However, Nrf2 might not directly be a substrate of ERK1/2. Instead, it is discussed that ERK1/2 phosphorylates the nuclear transcription co-activator CREB-binding protein (CBP) and that CBP enhances Nrf2 transcriptional response (35,36). In addition BeWo cells were stimulated with VEGF, and the phosphorylation status of ERK1/2 was analyzed to substantiate the activation status of the kinases (Fig.2A).

It is generally accepted that oxidative or electrophilic stress is a requirement for Nrf2 activation (19,20). Thus, it was assumed that this activation may occur via VEGF-induced NAD(P)H oxidase activation, since VEGF is supposedly utilizes ROS as a second messenger after VEGF receptor-2 stimulation (40). However, NAD(P)H oxidase inhibition had no effect on the VEGF-triggered Nrf2 activation so that oxidative stress can be excluded here (Fig 2 A, DPI, 2 F). In this current paper, it is shown that VEGF induces Nrf2 activation and that this activation occurs without oxidative stress stimulus. This means, in effect, that VEGF stimulation prevents oxidative stress. Hence, it can be hypothesized that decreased VEGF bioavailability during preeclampsia results in reduced basal defense against oxidative stress and a higher vulnerability of the cells towards oxidative damage.

For testing this hypothesis, BeWo cells were stimulated with VEGF before challenging these cells with H$_2$O$_2$ implying oxidative stress. As expected, VEGF-stimulated cells showed less cell death and lower vulnerability after oxidative stress treatment with H$_2$O$_2$ than that of the VEGF-unstimulated control cells (Fig. 3 A and C). These results clearly showed a protective effect of VEGF against oxidative stress, comparable to the described effects of EGF (41). To elucidate the role of Nrf2 in the protective effect of VEGF, a BeWo cell line (BeWo-shNrf2), that carried a stable transfected shRNA against Nrf2-mRNA, was utilized. This cell line was no longer able to activate the Nrf2/ARE-system (Fig. 1 B). Thus shRNA technology instead of dominant negative Nrf2 over-expression was applied, because shRNA only knocks down Nrf2. By contrast, dominant negative Nrf2 blocks the binding to ARE by competitive inhibition and thus blocks all factors having ARE affinity. In these Nrf2-deficient BeWo cells, VEGF no longer protects against oxidative stress, which infers that Nrf2 has a pivotal functions in cytoprotection mediated by VEGF (Fig. 3 B and D). These results indicate that cell death may be induced in villous trophoblasts following ROS exposure, and demonstrate the placental protective effect of VEGF.

As a transcription factor, Nrf2 unfolds its protective effect via up-regulation of genes coding for antioxidant enzymes. Therefore, it was studied if VEGF up-regulates the
expression of Nrf2 target genes in the applied system. Exemplarily, thioredoxin, thioredoxin-reductase 1 and HO-1 were sought, because these enzymes has been shown to be less expressed in the placenta of women suffering from preeclamptic (42-44). Supporting the proposed hypothesis, all three corresponding Nrf2 target genes were up-regulated in response to VEGF treatment (Fig. 1 D and F).

In particular, decreased gene expression for HO-1 and the resultant decline in CO-production has been shown to initiate pathological processes throughout gestation (40,42). Cudmore and collaborators demonstrated that the HO-1/CO pathway inhibits sFlt-1 and sEng release, providing compelling evidence for a protective role of HO-1 in pregnancy (45). Also Zhao et al. showed that deficiency in HO-1 is associated with elevation in maternal diastolic blood pressure and plasma sFlt-1 levels (46). Recently, Lin et al. showed that HO-1/CO is able to induce VEGF expression in myocytes (47). For the first time this current paper proves a positive feedback loop of VEGF inducing the expression of itself via Nrf2/HO-1 activation. A sketch of the assumed signalling pathway of VEGF-induced HO-1 expression via Nrf2 is shown in Fig. 5.

These findings also prove a protective role of the Nrf2/HO-1/CO system during pregnancy. In this context, HO-1 up-regulation via Nrf2 activation shows great potential to serve as an effective drug target in future pharmacological therapy for preeclampsia (48).

In conclusion, VEGF-induced Nrf2 activation prevents placental oxidative stress. Therefore, it can be hypothesized that decreased VEGF bioavailability during preeclampsia results in insufficient Nrf2 activation, reduced basal defense against oxidative stress and a higher vulnerability to placental oxidative cell damage. Resulting damage to the placenta causes disproportionate release of toxic placental factors that are manifested as preeclampsia and endanger maternal health.

Specific attempts to strengthen the fetal endogenous defense system against oxidative stress with Nrf2 inducer i.e sulforaphane, Methysticine or Andrographolide at an early gestation stage could prove to be a possible therapeutic option and may, in turn, reduce the risk of preeclampsia and associated perinatal complications.

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FOOTNOTES
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³The abbreviations used are: Nrf2, nuclear factor erythroid 2-related factor-2; VEGF, vascular endothelial growth factor; HO-1, heme oxygenase; CO, carbon monoxide; TRX, thioredoxin; TXNRD, thioredoxin reductase; ROS, reactive oxygen species; ERK, extracellular-signal-regulated kinases; H₂O₂, hydrogen peroxide.

FIGURE LEGENDES

FIGURE 1. VEGF₁₆₅ activates the Nrf2/ARE system. (A) Dual luciferase assay with BeWo cells transfected with the ARE-dependent luciferase reporter gene plasmid. VEGF₁₆₅ activates Nrf2 in a dose-dependent manner. 1 µM sulforaphane (SFN)-stimulation were used as positive control. (B, upper graph) Nrf2 was down-regulated by 90 % compared to control via stable transfected Nrf2 specific shRNA. (B, lower graph). VEGF stimulation of BeWo cells with Nrf2-knockdown failed to activate the Nrf2/ARE system in an ARE- Dual luciferase assay, so did SFN the positive control. (C) Western blots analysis to measure Nrf2 activation in response to VEGF-stimulation in BeWo cells. Nrf2 translocates into the nucleus in response to VEGF stimulation in BeWo cells. (D) Western blot analysis to measure the effects of VEGF on the expression of antioxidative stress genes in BeWo cells. Western blot shows upregulation of heme oxygenase-1, thioredoxin and thioredoxin reductase 1 protein expression (F) The density of corresponding bands and the loading control β-actin was measured and the ratio was calculated. The mean ±SEM. of three independent experiments is shown. Data are presented as fold induction after treatment compared with untreated cells (control = 1). p < 0.05, ** p < 0.005, *** p < 0.001, # p < 0.0001 versus control (One representative Western blot was shown; n=3).

FIGURE 2. VEGF₁₆₅ activation of Nrf2 is ERK1/2-dependent. (A) Dual luciferase Assay to analyze cell signalling of VEGF-mediated Nrf2/ARE activation. VEGF was added to the cell culture 30 min after addition of the kinase or NAD(P)H oxidase inhibitors [MEK1 inhibitor inhibitor PD98059 (1-50 µM), MEK1/2 inhibitor U0126 (1-10 µM), p38 inhibitor SB203580 ( 1-40 µM), PI3K inhibitor Wortmannin (0.1-5 µM) and NAD(P) oxidase inhibitor Diphenyliodonium chloride (DPI) ( 1-20 µM).
Data are presented as percentage fold induction of luciferase activity after treatment compared to control (treated cells without inhibitor, control = 100%).

(B) Immunoblot analysis utilising phospho-specific anti-ERK1/2 antibodies (pERK1/2) was used to measure ERK1/2 activation. BeWo cells were pretreated with either PD98059, or U0126 for 30 min, followed by treatment with VEGF$_{165}$ (10 ng/ml) for 1 h. The membrane was stripped and probed with anti-ERK1/2 antibodies for loading control (ERK1/2). The densities of corresponding pERK1/2 and ERK1/2 bands were measured and the ratio was calculated. The mean of three independent experiments is shown. Data are presented as fold induction after treatment compared with vehicle control (control = 1).

(D) The role of ERK1/2 on Nrf2 activation in response to VEGF treatment measured by Western blot. BeWo cells were pretreated with PD98059, or U0126 for 30 min, followed by treatment with VEGF$_{165}$ (10 ng/ml) for 1 h. Immunoblot from BeWo nucleus extracts with Nrf2 antibodies were used to analyse Nrf2 nucleus translocation. After Nrf2-immunoblotting the membrane was stripped and probed with anti-β-actin antibodies for loading control. Band intensity was calculated as the ratio of untreated control cells after normalization to the amount of loading control. Intracellular redox state levels of BeWo cells were measured using the fluorescent dye, H$_2$DCFH-DA. 4 beta-phorbol 12-myristate 13-acetate (PMA) 1 µM was used as a positive control. The positive effect of PMA was decreased using DPI (10 µM) 30 minutes before the addition of PMA. Data represented are the average of three separate experiments (mean± SEM).* p < 0.05, ** p < 0.005, *** p < 0.001, # p < 0.0001.
Figure 2

A

Relative ARE activity

B

pERK1/2

ERK1/2

VEGF_165 10 ng/ml

C

Relative intensity

D

Nrf2

β-actin

VEGF_165 10 ng/ml

E

Relative intensity

F

ROS production

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Figure 3

A

shControl

Relative viability

control  VEGF  VEGF +  VEGF -

B

shNrf2

Relative viability

control  VEGF  VEGF +  VEGF -

C

shControl

Relative cell death

control  VEGF  VEGF +  VEGF -

D

shNrf2

Relative cell death

control  VEGF  VEGF +  VEGF -

500 μM H₂O₂ + Fe²⁺
Figure 4

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)
Figure 5
Interplay between vascular endothelial growth factor (VEGF) and the nuclear factor erythroid 2-related factor-2 (Nrf2): implications for preeclampsia
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