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Research Article

Hyperbaric Oxygen Induced Vitamin D Receptor and ROS Responsive Genes: A Bioinformatic Analysis

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Keywords

- Reactive oxygen species
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Abstract

Background and Aim: Although reactive oxygen species (ROS) are often attributed to pathological outcomes, they do function as important modulators of many cellular processes involved in homeostasis and cell survival. Hyperbaric oxygen therapy (HBOT) initiates ROS production and signaling to promote angiogenesis, while limiting inflammatory and prothrombotic processes. In this study we set out to elucidate potential novel ROS-mediated, pro-angiogenic pathways.

Methods: Human umbilical cord endothelial cells (HUVECs) were subjected to standard HBOT parameters. HBOT-induced differential gene expression analyses were performed by coupling high-throughput RNA screening with bioinformatics.

Results: HBOT differentially regulated genes involved in a myriad of biological process, including a VD_3 1A hydroxylase/Vitamin D Receptor (VDR) signaling cascade that is both ROS responsive and pro-angiogenic.

Conclusions: HBOT regulates ROS responsive angiogenic genes, potentially in a VDR dependent manner.

ABBREVIATIONS

HBOT: Hyperbaric Oxygen Therapy; VDR: Vitamin D Receptor; ROS: Reactive Oxygen Species; CYP27B1: Cytochrome P450 Family 27 Subfamily B Member 1; ADCYAP1R1: Adenylate Cyclase Activating Polypeptide 1 (pituitary) Receptor Type I; ARHGAP6: Rho GTPase Activating Protein 6; NOS1: Nitric Oxide Synthase 1; SIRT6: Sirtuin 6; TYP: Tyrosinase

INTRODUCTION

Reactive oxygen species (ROS) are second messengers that serve many functions spanning immune response augmentation to cell cycle regulation. ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and hydroxyl radical (•OH), are byproducts of oxygen (O_2) metabolism that serve to maintain redox homeostasis in many physiological processes [1]. Redox regulation occurs via interplay between oxidative and anti-oxidative enzymes and processes. In endothelial cells (ECs), ROS are produced by enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase, and endothelial nitric oxide (NO) synthase (eNOS); and processes such as eNOS uncoupling and mitochondrial transport chain activity [1-3]. These are countered by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin-dependent peroxidase (TrxR2) and catalase [2,3].

NOXs are membrane bound enzymes that are composed of six subunits facing the extracellular space. The four isoforms expressed in vascular tissues are NOX1, NOX2, NOX4, and NOX5 [4]. NOXs couple electrons (e^-) from NADPH to O_2 producing reactive free radical superoxide anions by: NADPH + $2O_2$ ↔ $NADP^+ + 2O_2^- + H^+$. ROS produced by NOXs are reduced by SOD, which catalyzes the conversion of two O_2^- molecules into a molecule of H_2O_2, which is further reduced to water (H_2O) and O_2 by GPx, TRXR2, and catalase [3,5]. Mitochondria work in concert with NOXs to maintain redox homeostasis. ROS produced by NOX increase mitochondrial activity and ROS production; while mitochondrial ROS often increase NOX ROS instigating a feed forward cycle [2]. During aerobic metabolism, most O_2 is reduced

to H_2O in the mitochondria. However, e^- transfer through the mitochondrial respiratory chain, particularly at complexes I and III, offers the opportunity for early reduction of O_2 producing O_2^- instead of H_2O [6]. Mitochondrial enzymes such as pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, glycerol 3-phosphate dehydrogenase, or processes such as fatty acid beta-oxidation, also contribute to the production of O_2^- [6].

It is well established that prolonged oxidative stress causes vascular dysfunction and disease, such as atherosclerosis, leading to tissue ischemia. At the same time, the initiation of tightly regulated angiogenic pathways is a natural physiological response to tissue ischemia. Interestingly, the pharmacological reduction of ROS availability decreases angiogenesis by either activating ROS scavengers or SOD; or by inhibiting ROS production via NOXs [7]. This is in part due to down regulation of eNOS and consequent decrease in NO production [8]. The interplay between ROS and NO is required to maintain EC health, signaling and function [3]. ROS increases eNOS activity; and hence, NO production through caspase signaling [9]. Several studies have demonstrated the importance of ROS and NO signaling in angiogenesis [10-13]. For example, O_2^- and H_2O_2 induce angiogenesis by recruiting many cell types and mediators that facilitate differentiation, proliferation, migration, and adhesion [14]. Redox reactions also recruit pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), placental growth factor (PGF), platelet-derived growth Factor-B (PDGFB), transforming growth factor β (TGF- β), hypoxia inducible factors (HIFs) and angiopoietin-1 (ANG-1) [15-17].

Controlled oxidative treatments, such as hyperbaric oxygen therapy (HBOT), improve angiogenesis for certain pathologies such as impaired wound healing and ischemic heart disease. Currently, Undersea and Hyperbaric Medical Society has defined 13 indications for which HBOT is approved, including air/gas embolisms, CO poisoning, acute traumatic ischemia, and chronic wounds [18]. HBOT increases the partial pressure of oxygen in circulating plasma, which stimulates oxygen-dependent collagen matrix formation, a crucial step in wound healing and angiogenesis [19].

Mechanistically, HBOT stimulates the upregulation of NOX resulting in mitochondrial O_2^- production [6,17] and concomitantly reduces the circulating levels of pro-inflammatory cytokines, which are caused by ROS under stress conditions and wounded tissue [19]. This transiently-induced oxidative stress response not only upregulates growth hormones/factors; but, also activates and mobilizes stem/progenitor cells (SPCs) through transactivation of HIF-1 and 2 via thioredoxin reductase and thioredoxin [19,20]. Additionally, HBOT-induced ROS triggers the activation of signaling pathways that are involved in EC migration and invasion such as the mitogen-activated protein kinase (MAPK) family, c-Jun NH-2 terminal kinase, and extracellular regulated kinase (ERK) [21]. Although oxidative stress is often perceived as a precursor to pathology, acute oxidative stress does not necessarily result in oxygen toxicity. Oxidative stress, as characterized by elevated ROS, regulates many physiological processes involved in systems homeostasis, including angiogenesis in wounded or ischemic tissue. HBOT has proven effective in promoting ROS production and signaling necessary for neovascularization by instigating local EC proliferation and migration and systemically by recruitment and differentiation of stem progenitor cells (SPC) to form new vessels while limiting pro inflammatory and prothrombogenic mechanisms [19,20]. Although key mediators of angiogenesis, such as HIFs and VEGF [18], have been shown to be up regulated under HBOT, the molecular networks involved have not been delineated. Using high throughput RNA-sequencing and bioinformatics, this study uncovered $1\alpha,25(OH)_2D_3$/VDR signaling as a potential regulator of a novel redox responsive network involved in angiogenesis [19]. In this study, we set out to explore the effects of HBOT on the expression of genes involved in redox reactions to uncover potential novel pro-angiogenic-redox pathways. We coupled a high-throughput RNA expression analysis with bioinformatics to uncover potential ROS-responsive, pro-angiogenic genes differentially regulated following HBOT as an ROS stimulus. From this analysis, we uncovered the nuclear vitamin D transcription factor (VDR) as a potential HBOT-responsive initiator of a novel angiogenic cascade.

MATERIALS AND METHODS

Methods

Cell culture: HUVECs were cultured in Endothelial Cell Growth Medium (Sigma-Aldrich Cat# 211-500) supplemented with 15% (vol/vol) fetal bovine serum (FBS) and Primocin antibiotic (InvivoGen catalog no. ant-pm-1), and grown to confluency in a CO_2 incubator at 37°C and 5% CO_2.

HBOT: Cells were incubated in 2.0 atmospheres (atm) at 100% FiO_2 for 30 minutes in a model 1300 hyperbaric chamber (Sechrist industries) prior to lysis. Cells were placed on a water heat pad warmed to 37°C and monitored throughout the duration of the treatment via visual inspection.

RNA-sequencing: RNA was extracted with TRIzol reagent (ThermoFisher scientific cat#15596018) according to the manufacturer's instructions. Following isolation, the quality of the RNA analyzed with a Bioanalyzer prior to fragmentation with a Bioruptor UCD-200 sonicator to a length of 200 bp. Following sonication, the quality of the RNA and fragmentation length was analyzed a second time with a Bioanalyzer. cDNA was then prepared with the use of the Ovation RNA-Seq System V2 (NuGEN Cat# 7102) according to the manufacturer's instructions and sequenced on a illumine Hi Seq sequencing system. Sequence alignments and transcriptome analysis was conducted in R statistical environment and the system pipe R/Bioconductor software packages [22].

mRNA quantification: RNA was purified using TRIzol reagent. We converted 2µg RNA to cDNA using Promega reverse transcriptase according to the manufacturer's instructions. cDNA was then quantified via quantitative polymerase chain reaction (qPCR) using TaqMan Gene Expression Assays. Results were calculated using the delta-delta cycle threshold (ct) method using human actin as control. The following catalog numbers were used for the TaqMan assay.

Gene	Thermo-Fisher Scientific Cat#
CYP27B1	4351372

VDR	4331182
ADCYAP1R1	4331182
ARHGAP6	4331182
NOS1	4331182
SIRT6	4331182
TYR	4331182
ACTB	433118

Bioinformatics: EntroSolve (EntroSolve.com) was consulted for bioinformatics analysis. All bioinformatics computations were conducted in R statistical environment version 3.2.4. Gene ontology (GO) was conducted by downloading gene ontology information from BioMart and using text-mining algorithms included in the basic R package to identify proteins that are known to contribute to a subcellular process. Promoter sequences were obtained by querying BioMart for respective transcription start sites. The BSgenome.Hsapiens.UCSC.hg38 R package was then queried for promoter sequences to obtain DNA ranges +/- 2,000 base pairs from transcription start site. Pathway analysis was performed on the database that included putative VDR promoters using Gaggle software (http://gaggle.systemsbiology.net/docs/), String version 10 (http://string-db.org/), and Cytoscape (http://cytoscape.org/)

Statistical analysis: qPCR data are expressed as means ± SEM of at least three independent experiments. Comparisons of mean values between two groups were evaluated using a two-tailed Student's T-test or Mann-Whitney U-test. Unless otherwise indicated, p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Differential gene expression under HBOT

To investigate initiating transcriptome events that occur in response to increased ROS signaling, we subjected human umbilical cord endothelial cells (HUVECs) to HBOT at 100% F_iO_2 and 2 atm for 30 minutes. Following treatment, we conducted RNA-sequencing analysis then computed fold change values and significance levels of individual genes. Application of Bland-Altman was used to display expression analysis as an MA plot (Figure 1A). Following which, we determined the number of genes that have a p-value or False Discovery Rate (FDR) that meet a threshold cutoff of less than 0.05 (Figure 1B). Taking into account fold change levels in addition to the FDR, we categorized genes based on whether they had a fold change value less than 2.5 and an FDR greater than 0.05, a fold change greater than 2.5 and an FDR greater than 0.05, a fold change less than 2.5 and an FDR less than 0.05, or a fold change greater than 2.5 and an FDR less than 0.05, as represented by the Volcano Plot in (Figure 1C). Approximately 7,453 genes were identified to be significantly differentially expressed by at least a2.5 relative fold change under HBOT (Figure 1D).

Subset of ROS genes regulated by HBOT and their putative transcription factors.

Based on the importance of ROS signaling to HBOT-mediated

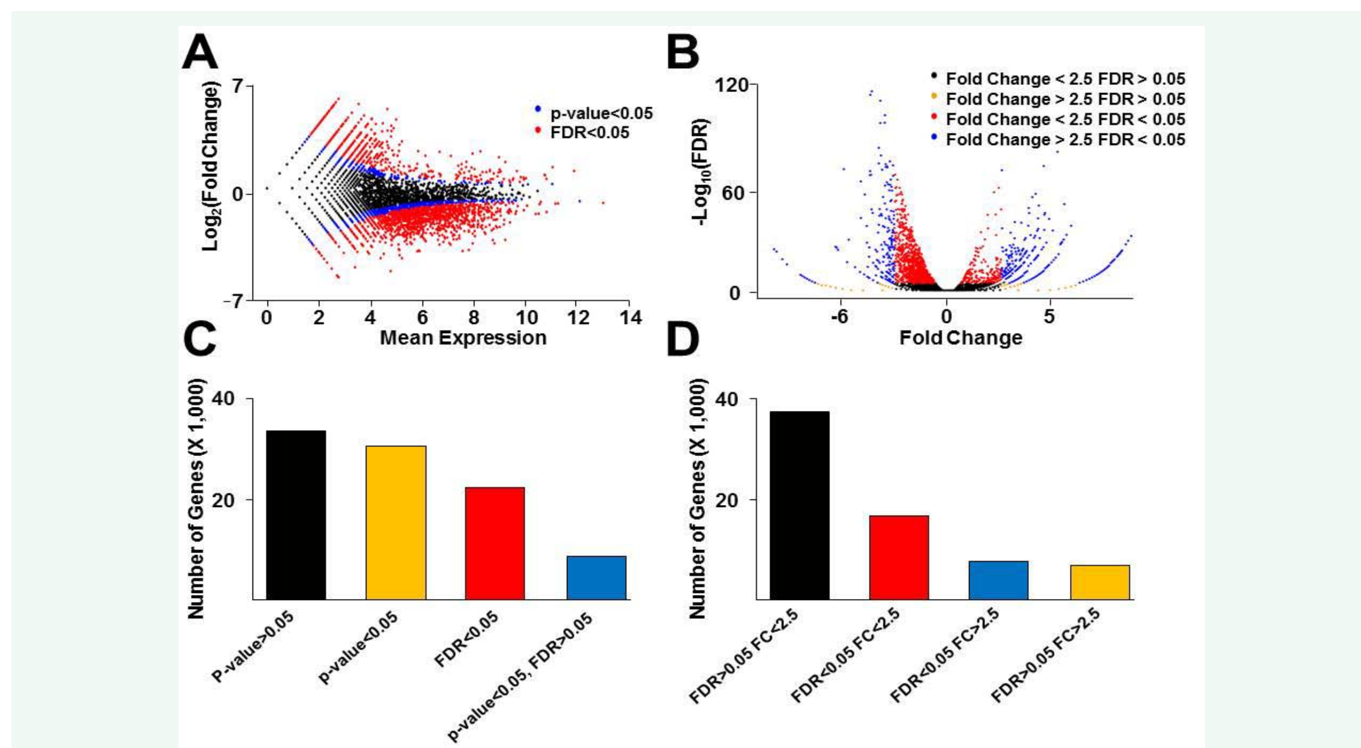
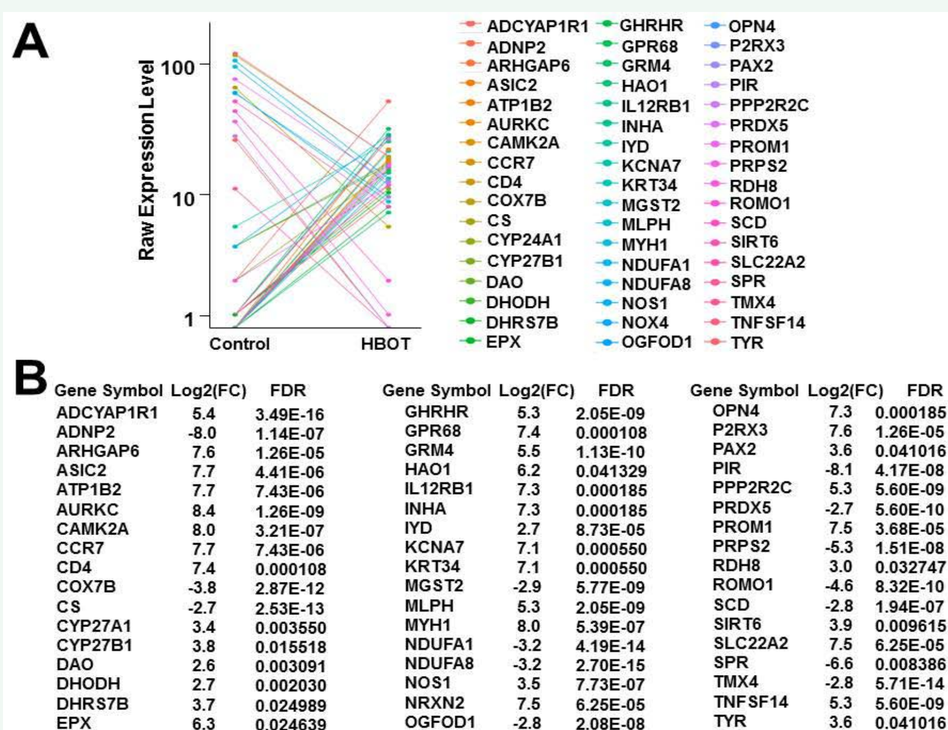


Figure 1 (A) MA plot illustrating RNA expression profile from HUVECs treated with 2 atm HBOT for 30 minutes. (B) Volcano plot representing RNA expression profiles from HUVECs treated with HBOT at 2 atm for 30 minutes. (C) Bar plot illustrating number of genes represented in specified significance range. (D) Bar plot illustrating the number of genes represented that have a specified fold change or FDR cutoff.

cellular response, we further classified genes that have a fold change greater than 2.5 and an FDR less than 0.05 based on their known functions in response to ROS-mediated signaling as annotated by Gene Ontology (GO) (http://geneontology.org). From this representative list, we identified 51 genes of interest (Figure 2A). Their relative expressions compared to control are listed in (Figure 2B). Next, we investigated putative transcription factors that may contribute to gene regulation of the 51 identified genes. We generated a function in R-statistical environment that utilizes consensus sequences scripted from the Motifmap database (http://motifmap.ics.uci.edu) to return the transcription factor consensus sequences that are present in a given DNA sequence. We then downloaded the DNA sequences +/-2,000 base pairs from the transcriptional start sites (TSS) of the 51 identified genes and determined the number of promoters containing a consensus sequence for each transcription factor in each of the 51 promoters (Figure 3A). We identified VDR to further investigate due to its implication in vitamin D and angiogenic signaling. We found the specific VDR DNA recognition sequence (Figure 3B) in 47 of the HBOT-induced ROS regulated promoters (Figure 3C). To gain insight into pathways that may be regulated by the VDR under HBOT-induced ROS, we queried the String database for 50 known and/or predicted genes associated with the 47 predicted VDR regulated genes and integrated these data into Gaggle and Cytoscape. Major nodes identified from the large database were pulled out and enlarged to allow visualization, which are depicted with a diamond shape (Figure 4B): adenylate cyclase activating polypeptide 1 (pituitary) receptor type I (ADCYAP1R1 or PAC1R), rho GTPase activating protein 6 (ARHGAP6 or RHOGAP6), nitric oxide synthase 1 (NOS1 or nNOS), sirtuin-6 (SIRT-6), and tyrosinase(TYR). These genes were revalidated using qPCR on RNA isolated from HBOT treated cell lysates along with cytochrome P450 family 27 subfamily B member 1 (CYP27B1) and the VDR due to their roles in vitamin D regulated pathways (Figure 4B).The role of CYP27B1,VDR and their predicted downstream targets in ROS-mediated angiogenesis.

CYP27B1also known as 25-Hydroxyvitamin D_3 1-alpha-hydroxylase (VD_3 1A hydroxylase) synthesizes the nuclear hormone 1alpha, 25-dihydroxyvitamin D_3(1α,25$(OH)_2D_3$), the active form of vitamin D_3, which binds to the VDR [23]. Recent studies have attributed 1α,25$(OH)_2D_3$ necessary to angiogenic signaling, especially in developing tissue [24]. CYP27B1 up regulation in HBOT may be an initiating factor in VDR regulation of ROS-dependent pro-angiogenic genes.

VDR is a ligand inducible nuclear transcription factor that binds 1α,25$(OH)_2D_3$ with high affinity. VDR targets many genes involved in cellular metabolism, bone formation, cellular growth and differentiation, and inflammatory response [25]. Significant to this study, current evidence suggests VDR signaling is vital to the modulation of angiogenesis as both endothelial cells (EC) and smooth muscle cell (SMC) are highly responsive to 1α,25$(OH)_2D_3$ [26]. Under pathogenic conditions, such as cancer, 1α,25$(OH)_2D_3$ is thought to play an anti-angiogenic role [27]. However, under normal physiological conditions or controlled induction of transient ROS, as occurs with HBOT, 1α,25$(OH)_2D_3$ is also involved in the promotion of angiogenesis [26]. For example, in ECs and SMCs, vitamin D improves eNOS expression and coupling to increase NO production while controlling ROS [28]. eNOS and NO



Gene Symbol	Log2(FC)	FDR	Gene Symbol	Log2(FC)	FDR	Gene Symbol	Log2(FC)	FDR
ADCYAP1R1	5.4	3.49E-16	GHRHR	5.3	2.05E-09	OPN4	7.3	0.000185
ADNP2	-8.0	1.14E-07	GPR68	7.4	0.000108	P2RX3	7.6	1.26E-05
ARHGAP6	7.6	1.26E-05	GRM4	5.5	1.13E-10	PAX2	3.6	0.041016
ASIC2	7.7	4.41E-06	HAO1	6.2	0.041329	PIR	-8.1	4.17E-08
ATP1B2	7.7	7.43E-06	IL12RB1	7.3	0.000185	PPP2R2C	5.3	5.60E-09
AURKC	8.4	1.26E-09	INHA	7.3	0.000185	PRDX5	-2.7	5.60E-10
CAMK2A	8.0	3.21E-07	IYD	2.7	8.73E-05	PROM1	7.5	3.68E-05
CCR7	7.7	7.43E-06	KCNA7	7.1	0.000550	PRPS2	-5.3	1.51E-08
CD4	7.4	0.000108	KRT34	7.1	0.000550	RDH8	3.0	0.032747
COX7B	-3.8	2.87E-12	MGST2	-2.9	5.77E-09	ROMO1	-4.6	8.32E-10
CS	-2.7	2.53E-13	MLPH	5.3	2.05E-09	SCD	-2.8	1.94E-07
CYP27A1	3.4	0.003550	MYH1	8.0	5.39E-07	SIRT6	3.9	0.009615
CYP27B1	3.8	0.015518	NDUFA1	-3.2	4.19E-14	SLC22A2	7.5	6.25E-05
DAO	2.6	0.003091	NDUFA8	-3.2	2.70E-15	SPR	-6.6	0.008386
DHODH	2.7	0.002030	NOS1	3.5	7.73E-07	TMX4	-2.8	5.71E-14
DHRS7B	3.7	0.024989	NRXN2	7.5	6.25E-05	TNFSF14	5.3	5.60E-09
EPX	6.3	0.024639	OGFOD1	-2.8	2.08E-08	TYR	3.6	0.041016

Figure 2 Raw expression levels (A) or fold change levels with associated FDR (B) of mRNA from HUVECs treated with HBOT at 2 atm for 30 minutes. Genes illustrated were selected due to their implications in ROS signaling.

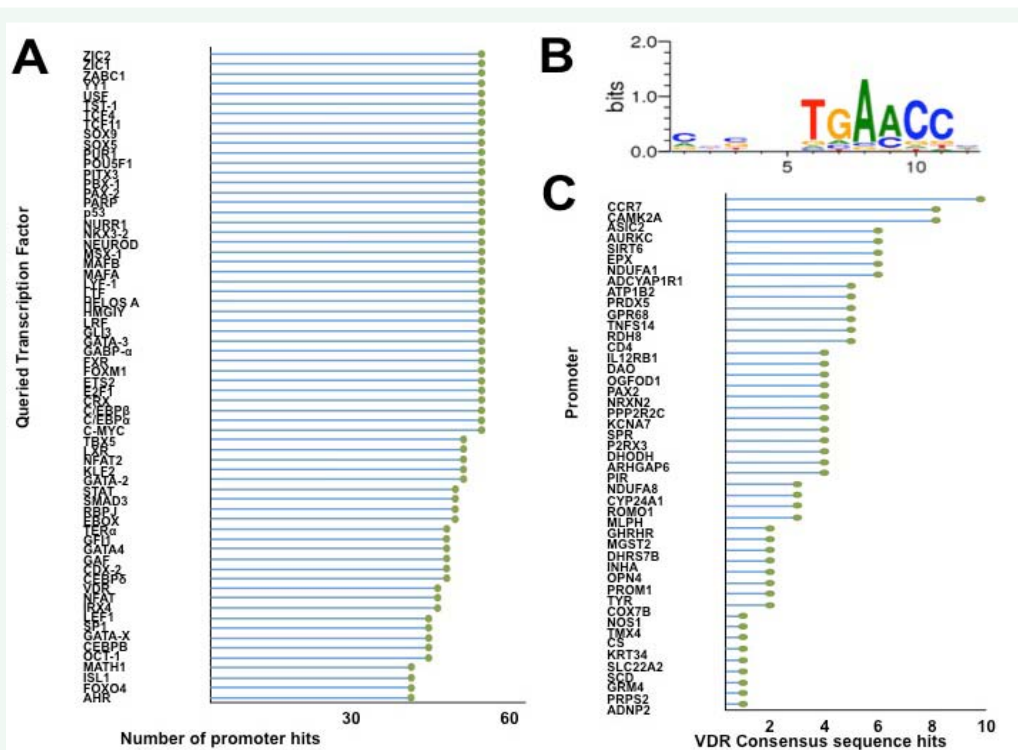


Figure 3 (A) Promoter sequences of the selected ROS genes were queried for the presence of transcription factor binding sites. The plot illustrated represents the number of promoters that contain at least one transcription factor consensus sequence. (B) Consensus sequence logo of the VDR transcription factor from the Motifmap database. (C) Number of times the VDR consensus sequence appears in the promoters of the selected genes.

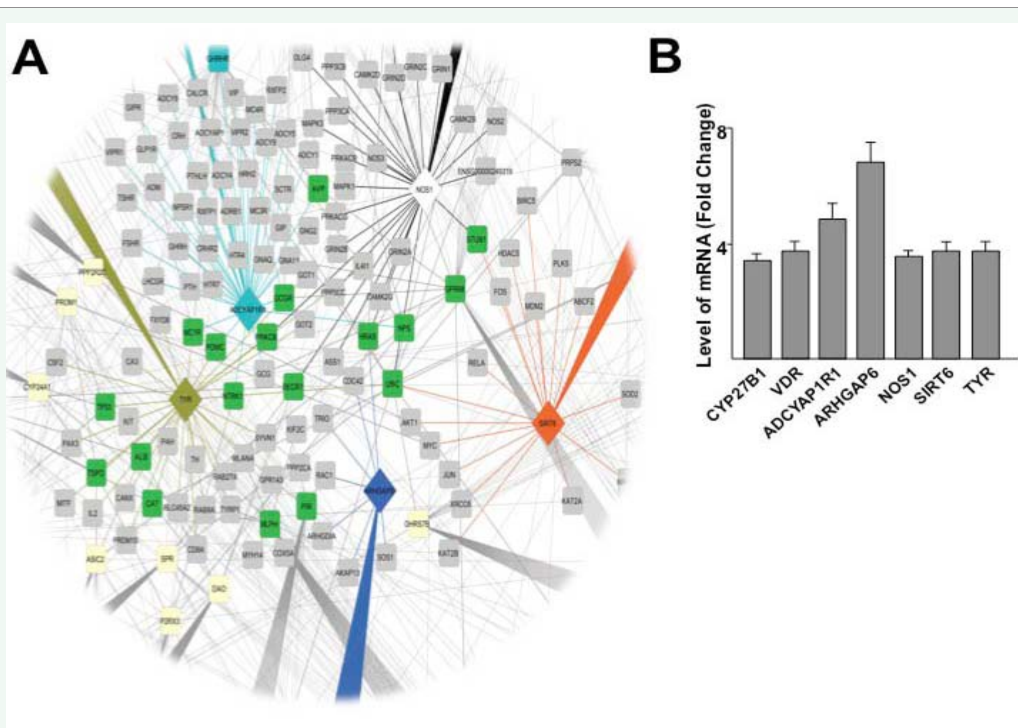


Figure 4 Cytoscape display of predicted VDR regulated, HBOT-induced ROS responsive genes. Central nodes are depicted as diamond shapes. Colors of lines indicate first order association congruent with each central node: light blue -ADCYAP1R1, dark blue – ARHGAP6, white – NOS1, orange – SIRT6, beige – TYR. First order-associated proteins are depicted in gray. Excluding central nodes, proteins with more than five associations are depicted in green (first order) and yellow (second order).(D) qPCR data representing fold change under HBOT compared to control, which was set at 1. All fold changes are significant with $p > 0.05$ and $N = 4$.

are well known for their role in angiogenesis. VDR signaling also promotes an angiogenic influence on endothelial progenitor cells (EPCs) through VEGF and matrix metalloproteinase 2 (MMP-2) regulation [29]. HBOT regulated VDR expression (Figure 4B) may prime a pro-angiogenic network of genes. As presented in (Figure 3C), the following HBOT regulated genes contain the VDR binding sequence within the promoters indicating their potential for VDR regulation.

ADCYAP1R1 (or PAC1R) is a membrane-associated receptor that mediates diverse biological actions of adenylate cyclase activating polypeptide 1 (PACAP). PACAP promotes pro-angiogenic effects and factors, such as EC proliferation and VEGF, respectively, both *in vivo* and *in vitro* [30]; while dysregulation of PAC1R-PACAP signaling impairs angiogenic responses in ECs [31]. PAC1R-PACAP signaling is activated by ROS, yet serves a regulatory role against hyper-physiological levels of ROS by increasing glutathione formation and reducing H_2O_2 ROS accumulation. This provides a protective effect against ROS-induced mitochondrial dysfunction, caspase 3 activation and stress-induced apoptosis [32]. PACAP further inhibits apoptosis by limiting $1\alpha,25(OH)_2D_3$dependent NF-κB ligand (RANKL) [33] anti-angiogenic influence [34] and by encouraging phosphorylation of anti-apoptotic ERK [35]. Therefore, HBOT transactivated PAC1R may serve to enhance angiogenesis by modulating ROS signaling toward necessary EC proliferation and differentiation as opposed to ROS-induced apoptosis.

ARHGAP6 (or RHOGAP6) is a cytoskeletal protein that activates RhoAGTPase to promote actin polymerization and remodeling [36]. Rho GTPases are constituents of activated NOX complexes and therefore participate in the generation of ROS (O_2^-) from O_2 [37]. Specifically, RhoA initiates the phosphorylation of the NOX subunit $p47^{phox}$, through the Rho-associated coiled-coil containing protein kinase 1 (ROCK)-p38MAPK pathway [38]. The RhoA-ROCK pathways are key to the process of angiogenesis at each step, which includes basement membrane permeability, EC proliferation and migration, cellular morphogenesis that leads to tube formation, recruitment of pericytes and vascular smooth muscle cells (VSMCs) necessary for vascular support [39]. Furthermore, RHOGAP6 and VDR induce phospholipase C (PLC) and enhance its activity [40,41] which has been shown to be important for vascular organization and stability during angiogenesis [42]. RHOGAP6 likely supports HBOT-induced angiogenic processes by modulation of ROS activation of both RhoA-ROCK and VDR pathways.

NOS1 (or nNos) synthesizes NO from L-arginine. nNOS is co-expressed with the endothelial eNOS (NOS3) in ECs, suggesting a possible role for nNOS endothelial function [44]. However, it has been shown to be predominately located in the nucleus while contributing to the basal level of NO production in ECs [45]. ROS signaling upregulates NOS expression, in part, through activation of NF-κB [46] but largely through $1\alpha,25(OH)_2D_3$, acting as a direct transcriptional regulator of all NO synthases [47]. Both nNOS and eNOS positively influence the transactivation of VEGF and angiogenesis [48]. Importantly, NO is necessary for revascularization of infarcted tissue [49]. nNOS-catalysis of NO production is more sensitive to ambient O_2concentration than eNOS, potentially revealing a significant role in HBOT-induced angiogenesis [50].

SIRT-6 is a histone deacetylase and an ADP-ribosyltransferase. SIRT-6 is activated under oxidative stress to ADP-ribosylate and activates poly ADP-ribose polymerase 1 (PARP-1) in DNA repair [51]. PARP-1 activation is a required component of signaling pathways involved in EC proliferation and angiogenesis [52]. Sirt-6 also potentially activates transient receptor potential cation channel subfamily M member 2 (TRPM2) by increasing ADP-ribose [53]. Activation of TRPM2 increases nuclear Ca^{2+} responses to regulate nuclear factor of activated T cells (NFAT), which transactivates angiopoietin-2 (ANG-2) [54], a key regulator in angiogenesis [55,56]. Notably, VEGF-induced ROS also activates TRPM2, which forms a complex with vascular endothelial (VE)-cadherin to secure EC junctions [57] exemplifying VE-cadherin's role in angiogenesis and neovascularization in ischemic tissue [58]. The SIRTs, including SIRT-6, are implicated in chromatin remodeling as histone deacetylases that transactivate VDR [59] to instigate angiogenesis and protect tissue damage in the presence of elevated H_2O_2 [50-62]. SIRT-6 likely plays a multifaceted role in HBOT-induced ROS responsive angiogenesis at both the transcriptional and cellular signaling levels.

TYR is a tyrosine hydroxylase and a dopa oxidase that catalyzes at least 3 steps in the pathway that converts tyrosine to melanin. TYR positively influences vascular regeneration and EPC recruitment after exposure to O_2 [63], potentially by catalyzing the oxidation of catecholamines. Interestingly, the intermediate products of tyrosine to melanin conversion differentially regulate angiogenesis relative to the degree of pigmentation of tissue (higher pigmentation correlates to reduced angiogenic response). For example, dopamine is predominately anti-angiogenic; whereas, norepinephrine and epinephrine are pro-angiogenic in ischemic tissue [64]. Furthermore, TYR activity increases ROS stimulating VEGF expression, proliferation and migration in ECs [65,66]. In regards to vitamin D, it has been shown to increase tyrosine levels in melanocytes and may have similar effects in ECs [67]. HBOT transactivation of TYR may indeed facilitate angiogenesis by providing optimal O_2 supply for pro-angiogenic intermediate production in tyrosine to melanin conversion.

CONCLUSION

In this study, we report that HBOT up regulates 51 novel ROS responsive genes identified through high-through put screening and bioinformatics. We also identified the $1\alpha,25(OH)_2D_3$/VDR pathway as a potential transcriptional regulator of 47 of these genes via a VDR consensus DNA binding domain located in their promoter. Specifically, we validated HBOT upregulation of CYP27B1, VDR, ADCYAP1R1, ARHGAP6, NOS1, SIRT6, and TYR using qPCR. These data support further investigation of molecular mechanisms and phenotypic outcomes of HBOT – ROS induced regulation of VDR and its downstream cascade in angiogenesis.

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