An oxidative DNA “damage” and repair mechanism localized in the VEGF promoter is important for hypoxia-induced VEGF mRNA expression

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Submitted 13 July 2015; accepted in final form 28 September 2015

An oxidative DNA “damage” and repair mechanism localized in the VEGF promoter is important for hypoxia-induced VEGF mRNA expression. Am J Physiol Lung Cell Mol Physiol 309: L1367–L1375, 2015. First published October 2, 2015; doi:10.1152/ajplung.00236.2015.—In hypoxia, mitochondria-generated reactive oxygen species not only stimulate accumulation of the transcriptional regulator of hypoxic gene expression, hypoxia inducible factor-1 (Hif-1), but also cause oxidative base modifications in hypoxic response elements (HREs) of hypoxia-inducible genes. When the hypoxia-induced base modifications are suppressed, Hif-1 fails to associate with the HRE of the VEGF promoter, and VEGF mRNA accumulation is blunted. The mechanism linking base modifications to transcription is unknown. Here we determined whether recruitment of base excision DNA repair (BER) enzymes in response to hypoxia-induced promoter modifications was required for transcription complex assembly and VEGF mRNA expression. Using chromatin immunoprecipitation analyses in pulmonary artery endothelial cells, we found that hypoxia-mediated formation of the base product 8-oxoguanine (8-oxoG) in VEGF HREs was temporally associated with binding of Hif-1α and the BER enzymes 8-oxoguanine glycosylase 1 (Ogg1) and redox effector factor-1 (Ref-1)/apurinic/apyrimidinic endonuclease 1 (Ape1) and introduction of DNA strand breaks. Hif-1α colocalized with HRE sequences harboring Ref-1/Ape1, but not Ogg1. Inhibition of BER by small interfering RNA-mediated reduction in Ogg1 and redox effector factor-1 (Ref-1)/apurinic/apyrimidinic endonuclease 1 (Ape1) binding to VEGF HRE sequences and blunted VEGF mRNA expression. Chromatin immunoprecipitation-sequence analysis of 8-oxoG distribution in hypoxic pulmonary artery endothelial cells showed that most of the oxidized base was localized to promoters with virtually no overlap between normoxic and hypoxic DNA data sets. Transcription of genes whose promoters lost 8-oxoG during hypoxia was reduced, while those gaining 8-oxoG was elevated. Collectively, these findings suggest that the BER pathway links hypoxia-induced introduction of oxidative DNA modifications in promoters of hypoxia-inducible genes to transcriptional activation.

Hypoxia; reactive oxygen species; DNA repair; transcriptional regulation

Hypoxia is a fundamental physiological and pathological stimulus mediating organogenesis during embryonic development, adaptive angiogenesis in situations where oxygen supply chronically fails to meet demands, ischemia-reperfusion injury in many organs, and, in the lung, hypertensive pulmonary vascular remodeling. Many of these effects of hypoxia are triggered by mitochondria-derived reactive oxygen species (ROS) that function to stabilize a key transcription factor driving hypoxia-induced mRNA expression, hypoxia inducible factor-1α (Hif-1α) (32). Hypoxia governs expression of ~500 genes, in part through interactions between Hif-1α and hypoxic response elements (HREs) located in gene regulatory sequences (2, 3, 24, 28).

Along with stabilizing Hif-1α, studies in organisms ranging in complexity from yeast to humans show that ROS generated in hypoxia cause transient oxidative DNA “damage” in nuclear DNA (9, 14, 23). The precise biological significance of hypoxia-induced oxidative nuclear DNA modifications has not been defined, but multiple lines of evidence suggest that they could be involved in transcriptional regulation (13). For example, oxidative base modifications evoked in rat pulmonary artery endothelial cells (PAECs) and smooth muscle cells by hypoxia and several other stimuli using ROS as second messengers are most prominent in the promoters of hypoxia-inducible genes; the 3’ guanine of the consensus Hif-1 DNA recognition sequence in HREs is conspicuously targeted for oxidative modification (14, 25, 35). Oxidatively modified VEGF HREs are also prominent in transcriptionally active mononucleosomes (30). More direct support for the idea that promoter base oxidation in hypoxia contributes to transcriptional regulation is derived from experiments modulating mitochondrial proximity to the nucleus (2). In these latter studies, although inhibition of hypoxia-induced perinuclear mitochondrial clustering failed to prevent either the cytoplasmic ROS stress or nuclear Hif-1α accumulation, it markedly reduced 8-oxoguanine formation in VEGF HREs, Hif-1α binding to the sequence, and VEGF mRNA expression. Thus formation of 8-oxoguanine in the VEGF HRE seems to be important for transcription complex assembly and VEGF mRNA formation. To distinguish this response, which may be incriminated in transcriptional regulation, from traditional DNA “damage” linked to toxicity and mutagenesis, we will hereafter refer to it as oxidative DNA “modifications.”

The finding that hypoxia-induced base modifications in the VEGF HRE are transient indicates that they are repaired (14), most likely by the four-step base excision DNA repair (BER) pathway. The first step is mediated by a DNA glycosylase, one of which is 8-oxoguanine glycosylase 1 (Ogg1) (17), which is responsible for detecting and excising the mutagenic base product 8-oxoguanine to leave an apurinic/apyrimidinic (AP) site. Ogg1 also displays DNA lyase activity and is thus able to create a single strand nick 5’ to the AP site formed by removal of 8-oxoguanine. Ogg1 is believed to “hand off” the AP site to the second enzyme in the pathway, redox effector factor-1 (Ref-1)/AP endonuclease 1 (Ape1). The AP endonuclease activity of the bifunctional protein is located on the carboxy...
terminal domain and functions to create a single-strand nick 3' to the AP site and prepare the site for insertion of a new base (10). The nick so created also engenders substantial sequence flexibility, which could be a determinant of promoter-nucleosome apposition or long-range DNA bending (5). The amino terminal domain of the molecule harbors Ref-1 activity and is responsible for redox activation of Hif-1 and other transcription factors and for assembly of coactivator proteins into the transcriptional complex (10, 34). After the third step, insertion of a new base by polymerase β, the final step, strand religation, is accomplished by DNA ligases 1 or 3 (17).

The requirement for Ref-1/Ape1 in assembly of the hypoxia-inducible transcriptional complex and the failure of Hif-1α to associate with the HRE when oxidative base modifications were suppressed raises the intriguing possibility that base modifications in HREs evoked by hypoxia-generated ROS are functionally linked to transcriptional activation via the BER pathway. To test this idea, here we applied chromatin immunoprecipitation (ChIP) assays in normoxic and hypoxic rat PAECs to determine whether hypoxia caused recruitment of BER enzymes to, and formation of strand breaks in, the VEGF HRE. Next, we used small interfering RNA (siRNA)-mediated knockdown of Ogg1 to explore the effect of BER suppression on Hif-1α and Ref-1/Ape1 recruitment to the HRE and on VEGF mRNA expression. Finally, we employed a newly developed ChIP-seq strategy to compare the disposition of 8-oxoguanine between the genomes of normoxic and hypoxic rat PAECs and to determine if accumulation of the oxidative base product was associated with hypoxia-induced changes in transcription.

METHODS

PAEC culture and VEGF mRNA expression. Rat PAECs isolated and cultured as described previously were used between passages 9 and 15 (2). For culture in normoxia or hypoxia, six-well tissue culture plates were placed in an incubator purged with either 21% or 2% O2, respectively, in normoxic and hypoxic cells was always within the dynamic range of the probe.

Chip analyses. As previously described (2, 8), ChIP analyses were performed using the commercially available ChIP-IT Express or ChIP-IT Express Enzymatic or Re-ChIP-IT kits, depending on the specific experiment (Active Motif, Carlsbad, CA), to determine the abundance of VEGF HRE sequences harboring the common base oxidation product, 8-oxoguanine (Millipore, Billerica, MA), and associated with Hif-1, Ref-1/Ape1, or Ogg1 (Novus Biologicals, Littleton, CO), and Ogg1 (Abcam, Cambridge, MA). Briefly, ~2×10⁶ PAECs were fixed with 1% formaldehyde (Sigma, St. Louis, MO) for 10 min and washed with ice-cold 1× PBS, and fixation reaction was terminated by addition of Glycine Stop Fix solution for 5 min. PAECs were then washed and collected in Cell Scraping Solution supplemented with 0.5 mM PMSF. Chromatin sheared to ~500-bp fragments by sonication or by enzymatic shearing was subsequently immunoprecipitated per manufacturer's instructions. Immunoprecipitated DNA and input DNA controls were amplified by qPCR using USB VeriQuest Fast SYBR Green qPCR Kit (Affymetrix, Santa Clara, CA) and primers specific for the VEGF HRE: 5'-GAAATGCTAGGGTAAATTTG-3' (forward) and 5'-GTGTCGATCTTCATACATAAGG-3' (reverse). As a negative control, a ~200-bp sequence of the VEGF promoter closer to the transcription start site but upstream from the damage-prone G-quadruplex region was amplified using the following primers: 5'-TGTCATACGCTACACCATCACC-3' (forward) and 5'-CCCTACCTCAAAAAAACACTATACC-3' (reverse).

Fluorescence microscopic detection of pan-cellular oxidant stress. Pan-cellular oxidative stress was monitored using a redox-sensitive green fluorescent protein (roGFP) probe previously described by our laboratory for assessment of endothelial cytoplasmic and nuclear ROS measurements (2). The probe is a double-cysteine-substituted GFP (C48S, S147C, Q204C, and S65T) so that it has a disulfide bond that reacts to oxidants/reductants in the same manner as GSSG and alters the emission intensity at 535 nm when excited at 400 and 490 nm. The 400-nm excitation/530-nm emission of roGFP increases with increasing oxidant levels, while the 485-nm excitation/530-nm emission decreases with increasing oxidant levels. The photometric fluorescence from two excitation wavelengths allows time-dependent quantitative determinations of oxidative stress in any part of the cell, irrespective of local concentration heterogeneity. In the present study, PAECs were cultured in normoxia or hypoxia for the indicated periods with ratiometric imaging of roGFP at 15-min intervals. After hypoxic exposure, cells were challenged with tert-butylhydroperoxide, followed by dithiothreitol, to define the dynamic range of the probe. Since the ratio of fluorescence emissions at 400 and 480 nm, respectively, in normoxic and hypoxic cells was always within the dynamic range of the probe.

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DNA affinity precipitation and Western immunoblot analyses of Hif-1α and its binding to an oligonucleotide model of the VEGF HRE. Western immunoblot assays of Hif-1α and actin proteins were performed as described previously (26), with the abundance of Hif-1α normalized to actin for graphical presentation and subsequent analyses. DNA affinity precipitation using a 65-mer sequence of the rat VEGF HRE, including the Hif-1 DNA recognition site and Western immunoblot analyses, were used to examine the intrinsic ability of Hif-1α to bind to an oligonucleotide model of the VEGF HRE. This procedure, too, was performed as described previously (2, 34).

ChIP-seq analysis. Input DNA samples, as well as DNA samples fixed and immunoprecipitated with 8-oxoguanine antibody, were reserved for library generation using the NEBNext Fast DNA Library Prep Set for Ion Torrent (New England BioLabs, Ipswich, MA) with subsequent sequencing using the Ion Personal Genome Machine, according to manufacturer’s protocol (Life Technologies). Individual reads (usually 36–100 bp) were mapped to the Ensembl Rattus reference genome using Bowtie standard default parameters (18), and a gene set enrichment analysis function was employed to identify regions with significant antibody binding. In a second analysis to ascertain if the region-specific loss or acquisition of 8-oxoguanine in hypoxia was associated with changes in transcriptional state of the gene, promoters enriched in 8-oxoguanine were aligned with our existing microarray data for hypoxic PAECs (8). The modified Kolmogorov-Smirnov test was then used to determine whether promoters gaining or losing 8-oxoguanine differed as a function hypoxia-induced changes in transcription, as previously described (33).

To determine whether transcription factor binding sequences were specifically enriched in 8-oxoguanine formed in promoters as a consequence of 3-h hypoxic exposure, all reads were scanned against known rat transcription factor-binding motifs (19) using an in-house algorithm and calculating normalized frequencies of representation per 1,000 bp. Proportional enrichments were expressed as the ratios of occurrence in hypoxic relative to normoxic data sets for each transcription factor binding sequence.

RESULTS

As anticipated from previous observations, Fig. 1, A and B, shows that the onset of hypoxia-induced VEGF mRNA expression was associated with rapid but transient accumulation of 8-oxoguanine in VEGF HRE sequences, as determined by qRT-PCR and ChIP analyses, respectively. Data in Fig. 1, C–F, also demonstrate that the hypoxia-induced formation of 8-oxoguanine in VEGF HRE sequences was accompanied by recruitment of the master regulator of hypoxia-induced transcription in PAECs, Hif-1α, the bifunctional DNA glycosylase and lyase Ogg1, the transcriptional coactivator and AP endonuclease Ref-1/Ape1, and formation of strand breaks in the sequence, respectively, as determined by ChIP analyses.

We next determined whether Hif-1α coimmunoprecipitated with sequences binding the BER enzymes Ogg1 and Ref-1/Ape1. PAECs were cultured in normoxia or hypoxia for 3 h, after which they were subjected to two-step ChIP analyses: the first step immunoprecipitated either Hif-1α, Ogg1, or Ref-1/Ape1.

Fig. 1. Temporal relationships between hypoxia (HYP)-induced VEGF mRNA expression (A) and formation of 8-oxoguanine (B), association of HYP inducible factor-1α (Hif-1α; C), the base excision DNA repair (BER) enzymes 8-oxooguanine glycosylase 1 (Ogg1; D), redox effector factor-1 (Ref-1/Ape1) (E), and formation of strand breaks (F) in the VEGF hypoxic response element (HRE). Values are means ± SE; N = 4–6 for each time point. *Different from normoxia (NORM) at P < 0.05.
Ape1 to verify association of the transcription factor and BER enzymes with HRE sequences, whereas the second step determined whether Hif-1α colocalized with either Ogg1 and/or Ref-1/Ape1-harboring HRE sequences. As shown in Fig. 2, Hif-1α colocalized with HRE sequences binding Ref-1/Ape1; Hif-1α was undetectable on HRE sequences binding Ogg1.

Additional experiments sought to determine whether recruitment of BER enzymes to the VEGF HRE was necessary for Hif-1α-DNA binding and for VEGF mRNA expression. Our strategy was to disrupt prosecution of base excision repair using siRNA to knock down the first enzyme in the four-step cascade, Ogg1, and then evaluate the effects in terms of VEGF mRNA expression, quantified by qRT-PCR, and 8-oxoguanine formation in, and Hif-1α and Ref-1/Ape1 association with, the VEGF HRE, as determined using ChIP assays. Transfection agent-treated or Ogg1 knockdown PAECs were cultured in normoxia or hypoxia for 3 h before isolation of chromatin and RNA for ChIP and qRT-PCR analyses, respectively.

An siRNA knockdown strategy previously reported by our laboratory was used to reduce the cellular abundance and activity of the DNA glycosylase initiating base excision repair of 8-oxoguanine, Ogg1 (31). In our laboratory’s earlier work, Ogg1 mRNA was reduced by ~85%, and total cell glycosylase activity directed against 8-oxoguanine was suppressed by ~60%. In the present study, mRNA abundance was reduced by 80 ± 5% (N = 4) relative to transfection agent-treated cells (not shown). As additional confirmation of the efficacy of the knockdown strategy, results of ChIP analysis of 8-oxoguanine displayed in Fig. 3A showed that, as expected, Ogg1 knockdown increased the abundance of the base oxidation product detected in VEGF HRE sequences in hypoxic cells relative to transfection agent-treated PAECs. To determine if siRNA-mediated Ogg1 knockdown impacted upstream events in hypoxic transcriptional signaling, we also evaluated hypoxia-induced ROS production using quantitative roGFP fluorescence. As shown in Fig. 3B, while there was a trend toward reduced roGFP signals in hypoxic PAECs depleted of Ogg1 compared with transfection agent-treated cells, differences did not reach statistical significance. We next used Western immunoblot analysis to determine whether the rise in Hif-1α evoked by hypoxia was blunted in cells depleted of Ogg1. As shown in Fig. 3C, there were no differences between hypoxia transfection agent-treated and Ogg1-depleted PAECs in terms of the abundance of this critical hypoxia-inducible transcription factor. Finally, DNA affinity precipitation and Western immunoblot assays were used to determine if siRNA-mediated Ogg1 knockdown impacted the intrinsic ability of Hif-1α to bind to its recognition sequence in an oligonucleotide model of the VEGF HRE. As displayed in Fig. 3D, the ability of the transcription factor to associate with an oligonucleotide sequence of the VEGF HRE was not impaired in Ogg1-depleted cells. Thus, as expected, whereas siRNA-mediated reduction in Ogg1 increased the level of 8-oxoguanine detected in the endogenous VEGF HRE, it was not associated with marked changes in core upstream signaling events in hypoxic transcription; namely ROS production, cellular Hif-1α accumulation, or the intrinsic ability of Hif-1α present in nuclear extract to bind DNA.

The effect of siRNA-mediated knockdown of Ogg1 on VEGF mRNA expression is shown in Fig. 4A. Ogg1 depletion was associated with significant attenuation of the hypoxia-induced rise in mRNA abundance for the growth factor. Perhaps more interestingly, when the Ogg1-mediated step in base excision repair was suppressed, two key members of the hypoxia-inducible transcriptional complex, Hif-1α (Fig. 4B) and Ref-1/Ape1 (Fig. 4C), failed to associate with the HRE sequence to the same extent as observed in transfection agent-treated PAECs.

The foregoing observations implied that recruitment of base excision repair enzymes to the VEGF HRE initiated by hypoxia-induced oxidative base modifications is important for VEGF mRNA transcription. In an initial effort to explore whether this concept could be generally applicable to hypoxic transcriptional regulation, we aligned the outcome of ChIP-seq assessment of the genomewide disposition of 8-oxoguanine with genes listed in the Ensembl Rattus reference genome. Out of the 22,414 protein-coding genes listed, 2.5% were immunoprecipitated by an antibody to 8-oxoguanine in normoxia, whereas only 0.9% genes immunoprecipitated with the oxidative base damage antibody in hypoxia. The distribution of 8-containing sequences between promoter and coding regions is shown in Fig. 5A. The majority of 8-oxoguanine-harboring sequences in both normoxic and hypoxic data sets was localized in promoters. Interestingly, there was virtually no overlap between experimental groups; that is, the immunoprecipitated sequences, most prominent in promoters but present to a lesser extent in coding sequences, originating from normoxic cells were almost entirely different from those immunoprecipitated from hypoxic PAECs.

In a further effort to explore the association between 8-oxoguanine disposition and transcription in hypoxia, we determined whether loss or acquisition of oxidized guanine in promoter regions was associated with changes in abundances of mRNA transcripts of the hypoxic transcriptome. Using our laboratory’s previously reported microarray analysis of genes differentially expressed in normoxic and hypoxic PAECs (8), we were able to align 61.7% of the 507 differentially regulated transcripts with promoter sequences harboring 8-oxoguanine in either normoxia or hypoxia. As shown in Fig. 5B, expression of the 120 genes whose promoters did not contain 8-oxoguanine

![Fig. 2. Two-step chromatin immunoprecipitation (ChIP) analysis of Hif-1α with HRE sequences simultaneously associating with either Ogg1 or Ref-1/Ape1. The first immunoprecipitation step detected VEGF HRE sequences associating with either Hif-1α, Ogg1, or Ref-1/Ape1, whereas the second step determined whether Hif-1α colocalized on HRE sequences simultaneously associating with either Ogg1 or Ref-1/Ape1. Note the presence of Hif-1α on DNA sequences immunoprecipitating with an antibody to Ref-1/Ape1, but not with an antibody to Ogg1. Gel images are representative of three experiments. N, NORM; H, HYP.](image-url)
in normoxia but acquired the base oxidation product in hypoxia was increased compared with hypoxic cells. In contrast, the 192 promoters that harbored 8-oxoguanine in normoxia but lost the base oxidation product after 3 h of hypoxia were associated with genes that almost uniformly decreased mRNA expression on hypoxic exposure.

Finally, we determined whether regulatory promoter regions in the hypoxic data set were enriched in 8-oxoguanine relative to the same DNA sequences from normoxic cells. The outcome of this analysis is presented in Table 1 and shows that promoter sequences known to bind a number of different transcription factors, including HREs associating with Hif-1α and Hif-2α, as well as other putative regulatory sequences previously shown to be “hot spots” for hypoxia-induced oxidative base damage [e.g., G-quadruplexes (8)] were enriched in 8-oxoguanine after hypoxic exposure compared with the same sequences from normoxic PAECs.

**DISCUSSION**

Over the past two decades, substantial evidence has accumulated to show that oxidative DNA “damage” occurs as a consequence of hypoxic exposure in species ranging in complexity from yeast to cells from rodents and human subjects (2, 3, 24, 28). Apart from the obvious implications, that such oxidative damage could lead to genomic instability, perhaps related to acquisition of mutations, the reasons why hypoxia engenders oxidative modifications in the nuclear genome have remained speculative. Indirect evidence, though, suggests that the targeted introduction of oxidative base lesions in functionally relevant DNA sequences could contribute to transcriptional regulation. For example, the oxidative modifications are prominent in HREs in hypoxia-inducible genes, particularly in the vicinity of Hif-1 DNA recognition sequences (25). They also are conspicuously localized in transcriptionally active mononucleosome structures (30). In what is perhaps the most persuasive support for the concept, we recently reported that mitochondria clustering in the perinuclear region during hypoxic exposure serves to create a ROS-enriched nuclear signaling domain associated with introduction of base modifications targeted to HRE sequences in hypoxia-inducible genes. When the nuclear ROS stress was suppressed by inhibition of perinuclear mitochondrial clustering, base modifications were not formed in the HRE, Hif-1α failed to bind to the sequence, and accumulation of VEGF mRNA was attenuated (2).

While the above observations suggest that targeted oxidative base modifications could contribute to transcriptional regulation in hypoxia, the mechanism linking base damage to gene expression is unknown. A clue to this relationship may lie in the observation that the lesions are transient, thus suggesting that they are repaired despite persistent hypoxic exposure (14, 25). Accordingly, the present study tested the hypothesis that recruitment of the BER pathway, the key process repairing oxidative DNA damage, was necessary for transcription com-

![Fig. 3. A: abundance of 8-oxoguanine detected by ChIP analysis of the VEGF HRE from NORM and HYP pulmonary artery endothelial cells (PAECs) treated with transfection agent alone (TA), or small interfering RNA (siRNA) to affect Ogg1 depletion. B: HYP-induced, redox-sensitive green fluorescent protein (roGFP)-detected oxidant stress expressed as a function of the NORM baseline (designated as “time 0”) in TA-treated and Ogg1-depleted (siRNA) PAECs. C: abundance of HIF-1α detected by Western immunoblot analysis in NORM and HYP cells treated with TA alone or with siRNA to deplete Ogg1. Representative Western blots are shown at the top, and pooled data are depicted at the bottom. D: representative DNA affinity precipitation (DNAP) and Western immunoblot analysis for HIF-1α present in nuclear extract derived from TA-treated or Ogg1-depleted PAECs binding to an oligonucleotide model of the VEGF HRE. Note that Ogg1 depletion is associated with a rise in HYP-induced 8-oxoguanine formation in the VEGF HRE, but fails to impair HYP-induced oxidant stress, HIF-1α abundance, or HIF-1α binding to the VEGF HRE oligonucleotide. Values are means ± SE. With the exception of the DNA affinity/Western immunoblot analysis, which was representative of 3 experiments, N = 4–6 for each data set. *Different from NORM at P < 0.05. **Different from HYP and NORM at P < 0.05.](http://ajplung.physiology.org/)

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complex assembly on the VEGF HRE and for VEGF mRNA expression. Several observations reported herein support this idea. First, we found that the first two enzymes in BER, Ogg1, and Ref-1/Ape1, and the BER intermediate, strand breaks, accumulated in HRE sequences in a manner that was temporally related to VEGF mRNA expression. The degree of variability around the mean values for Ogg1 recruitment and strand break formation was substantial; we attribute this to the notion that lesion repair rate may be temporally uneven across cells in culture, much like the variable pattern of oxidative base damage previously reported for G-quadruplex sequences in hypoxic PAECs (8). As discussed subsequently, the uneven progression of BER enzyme recruitment and strand break formation could provide clues as to the biological significance of this lesion formation and repair pathway.

We also found that a master transcriptional regulator in hypoxia, Hif-1α, associated not with HRE sequences binding Ogg1, but rather with sequences harboring the bifunctional transcriptional coactivator and BER enzyme, Ref-1/Ape1. This relationship between Hif-1α and Ref-1/Ape1 may also point to functional significance; the Ref-1 domain of the protein is required for proper maintenance of redox state and DNA binding activities of a number of transcription factors, including Hif-1α (10). Indeed, Ref-1/Ape1 seems to exist in the nucleus as part of a multiprotein complex with Hif-1α under hypoxic conditions (34). The Ape1 domain of the protein processes and creates a single-strand break at AP sites after

Fig. 4. A: effect of treatment with TA or siRNA to deplete Ogg1 (siRNA) on VEGF mRNA expression in NORM and HYP PAECs, as assessed by quantitative RT-PCR. ChIP analyses are shown of Hif-1α (B) and Ref-1/Ape1 (C) association with the VEGF HRE in NORM and HYP cells treated with TA or depleted of Ogg1 using siRNA. Values are means ± SE; N = 4–6 for each experimental group. *Different from NORM at P < 0.05.

Fig. 5. A: pie chart depicting ChIP-seq analysis of distribution of 8-oxoguanine (8-oxoG) between promoters and coding regions in genes from NORM and HYP PAECs. Nos. denote the no. of promoter or coding regions harboring 8-oxoG. Note particularly the preponderance of 8-oxoG in promoter regions and the fact that there were very few promoter or coding regions common to both NORM or HYP data sets. Data are representative of 3 separate ChIP-seq analyses. B: scatter diagram depicting relation between genes that acquired or lost 8-oxoG in HYP and gene expression in HYP. *The two data sets differed significantly at P < 0.05, as determined by the modified Kolmogorov-Smirnov test.
spontaneous base loss or after oxidized base removal by a DNA glycosylase like Ogg1 (10, 17). While the nick so produced may be important for insertion of the new base, it also engenders substantial sequence flexibility (5) and, if occurring in response elements, such as shown here, could play a role in enabling long- or short-range, energetically efficient DNA bending (27), or in relieving torsional stresses incurred by the actions of RNA polymerase at the transcription start site (6, 11, 20). Defining the specific roles of Ref-1/Ape1 in hypoxia-induced VEGF expression will require different strategies than those used herein, but the present data point to the intriguing concept that introduction of targeted base modifications in hypoxia could function to localize these two important activities of Ref-1/Ape1 in close proximity to its key transcriptional regulatory partner, Hif-1α, and its consensus binding sequence on the HRE.

Depletion of Ogg1 using siRNA did not impair core elements of ROS signaling in hypoxia; ROS generation, Hif-1α accumulation, and the intrinsic ability of Hif-1α to bind the HRE all proceeded with little decrement in Ogg1-depleted cells. In addition, and as expected, 8-oxoguanine accumulated to a greater extent in VEGF HRE sequences in Ogg1-depleted PAECs than in wild-type cells. Most importantly, the exaggerated accumulation of 8-oxoguanine was associated with diminished Hif-1α and Ref-1/Ape1 association with the VEGF promoter HRE and suppression of VEGF mRNA expression. We suspect that the failure of Hif-1α and Ref-1/Ape1 to bind to the HRE can be attributed to either the persistence of the oxidized base and/or the inability to prosecute the well-documented “hand-off” of the partially processed lesion from Ogg1 to Ref-1/Ape1 (10, 17). Whatever the specific mechanism by which transcription complex assembly is impaired, however, these findings suggest that activation of BER at the VEGF HRE in response to hypoxia-induced formation of 8-oxoguanine is required for high-fidelity transcription of the gene.

An important contribution of this work was the development of a ChIP-seq strategy for mapping the disposition of 8-oxoguanine on a genomewide scale in normoxic and hypoxic PAECs. A key part of our analysis was restricted to the hypoxic transcriptome, examined in this cell type by us and other groups several years ago (8). Our focus on the hypoxic transcriptome was motivated by the intent to explore the likelihood that the base damage-and-repair mechanism just described played a role in hypoxic transcription that extended beyond the VEGF gene. To our knowledge, this is the first time that genomewide base damage has been assessed on a sequence-specific basis, and the results are of interest for several reasons. First, the oxidative base modifications were predominantly located in promoters and enriched in functionally significant responsive sequences, findings that may be relevant to recent reports showing that the vast majority of somatically acquired sequence variation in human disease is located in regulatory regions (22). Since oxidative base damage is a documented precursor to mutation, the conspicuous localization of “damage” in promoters could point to ROS generated in

Table 1. Selected transcription factor binding sequences and functionally-relevant motifs enriched in 8-oxoG in hypoxia relative to normoxia

<table>
<thead>
<tr>
<th>Transcription Factor/Functional Motif</th>
<th>Fold Enrichment in Hypoxia</th>
<th>Putative Function(s)</th>
</tr>
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<tbody>
<tr>
<td>Hif-1, Hif-2</td>
<td>3.84, 12.20</td>
<td>Master regulators of hypoxic transcription by binding to HRE</td>
</tr>
<tr>
<td>Bach1</td>
<td>30.93</td>
<td>Competes with Nrf2 for binding to the antioxidant response element</td>
</tr>
<tr>
<td>c-Myc, N-Myc</td>
<td>21.00, 17.38</td>
<td>Binds to Ebox domains to regulate growth, differentiation, apoptosis, etc.</td>
</tr>
<tr>
<td>CTCF</td>
<td>32.91</td>
<td>Regulates 3D structure of chromatin by binding consensus sequence on DNA to form chromatin loops</td>
</tr>
<tr>
<td>E2A</td>
<td>21.77</td>
<td>Modulates Notch signaling by binding to Ebox domains</td>
</tr>
<tr>
<td>SOX4, SOX5, SOX10</td>
<td>3.02, 3.26, 5.50</td>
<td>Binds to consensus DNA sequences to affect DNA bending required for development and cell fate decisions</td>
</tr>
<tr>
<td>TCF11:MaF/G</td>
<td>32.22</td>
<td>Binds to a subclass of AP1 DNA binding sites to suppress transcription</td>
</tr>
<tr>
<td>G-quadruplex</td>
<td>7.32</td>
<td>An SP-1 binding, non-B-form sequence found in many promoters; may buffer transcription-related torsional stress</td>
</tr>
</tbody>
</table>

All reads immunoprecipitating with an antibody to 8-oxoguanine were scanned against known rat transcription factor-binding motifs (19) using an in-house algorithm. Frequencies of representation were normalized per 1,000 bp, and the “Fold Enrichment in Hypoxia” is expressed as the ratios of occurrence in hypoxic relative to normoxic data sets for each transcription factor binding sequence. Hif, hypoxia inducible factor; HRE, hypoxic response element; 3D, three-dimensional; AP1, activator protein 1.

![Fig. 6. Proposed mechanism whereby HYP-induced formation and repair of oxidative base modifications in regulatory sequences govern the rate of transcriptional cycling and thereby mRNA abundance. When the density of regulatory sequence base damage is elevated by HYP-induced oxidative stress, BER enzymes are recruited to the sequence at a greater rate to restore and retain normal sequence integrity. In contrast, in the absence of ROS stress targeted at regulatory sequences, the rate of DNA repair enzyme recruitment to the sequence is lower. The rate of DNA damage and BER-mediated repair is envisioned to be a determinant of transcription complex assembly/stability and thereby directly impact the rate of transcriptional cycling and mRNA accumulation. See text for additional details.](http://ajplung.physiology.org/)

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the context of transcriptional signaling as proximate initiators of acquired sequence variation. Second, we found that the promoter base modifications harbored by many genes in normoxia were absent in hypoxia, and that an almost distinct set of genes lacking base modifications in normoxia acquired them on transition to a hypoxic environment. Hypoxia, therefore, seems to be associated with gene-specific mechanisms for introducing and/or removing oxidative base modifications. Finally, there was a close relation between hypoxia-induced changes in transcriptional state and the loss or acquisition of promoter base modifications: those that lost base damage in hypoxia generally displayed reduced transcription, whereas those that gained base damage were generally upregulated. We believe this comprises provocative evidence that the BER-related mechanism employed by hypoxia to activate VEGF mRNA expression could be applicable to other genes in the hypoxic transcriptome. Further studies, perhaps facilitated by the ChIP-seq strategy described herein, will be required to rigorously explore this concept.

In light of the above observations, we advance a provisional model for the mechanism by which hypoxia-induced oxidative base modifications regulate expression of VEGF and perhaps other genes in the hypoxic transcriptome. As shown in Fig. 6, we envision that the promoter DNA “modifications and repair” pathway initiated by hypoxia-generated ROS and accomplished by the BER pathway, respectively, functions in transcriptional cycling. When targeted ROS stress is high, the modification and repair cycle must be rapid to prevent persistence of the damage and the inherent threat of mutation or transcriptional dysfunction. After the oxidative base lesion is excised by Ogg1, Ref-1/Ape1 complexed with Hif-1α and perhaps other transcriptional coactivators is recruited to the site as a prerequisite to transcription initiation. Based on previous reports (5), we suspect that, along with its contribution to BER, Ref-1/Ape1-mediated strand cleavage also serves to modulate local or long-range sequence topology and flexibility to facilitate transcription complex assembly. Subsequently, polymerase β-mediated new base insertion and strand relegation by DNA ligase are postulated to affect transcriptional inactivation, thereby restoring integrity of the response element for another round of activation. Rapid on-off cycles, driven by persistent oxidant stress targeted at the responsive sequence would, in the face of an unchanged rate of mRNA degradation, lead to mRNA accumulation. Conversely, when the ROS stress directed against a specific promoter sequence is low, a reduced rate of damage and repair cycling would engender an attenuated rate of mRNA accumulation. Furthermore, we speculate that the variability characterizing associations between the BER enzyme, Ogg1, and the BER intermediate, strand breaks, and the VEGF HRE (see Fig. 2) reflect the differing stages of lesion repair displayed by a heterogeneous population of cultured PAECs. Although the concept of transcriptional cycling is already established (7), our model adds the interesting feature that oxidative DNA modifications and ensuing repair are critical determinants of the cycling rate.

In summary, the findings reported herein confirm that the hypoxia-induced ROS stress in PAECs causes base modifications targeted to the VEGF HRE. To this, they suggest that the base modifications so produced are accompanied by recruitment of BER enzymes to, and formation of strand breaks in, the sequence. Because suppression of BER by depletion of the initial enzyme in the cascade forestalls transcription complex assembly and attenuates VEGF expression, these findings are consistent with a model in which base modifications and repair comprise a molecular link to the initial oxidant stress and transcriptional regulation. It should be noted that other systems display similar features. For example, estrogen-induced expression of the pS2 and cMyc genes also seems to require controlled DNA damage and repair, but in these instances the damage is initiated by a DNA-bound lysine demethylase, LSD1, rather than mitochondria-generated ROS (27). The involvement of the BER pathway has not been established in estrogen-induced transcriptional signaling, and the potential role of the damage and repair pathway in the estrogen-regulated transcriptome has not been explored. It is also appropriate to acknowledge that the findings presented herein are at least superficially similar to reports by others, suggesting that topoisomerase I or II initiate double-strand DNA cleavage required for changes in chromatin conformation and transcriptional activation of the pS2 gene in response to estrogen in MCF7 cells or activity-induced early response gene expression in primary cultures of neurons (15, 16, 29). Finally, Ogg1 in complex with its excision product, 8-oxoguanine, has recently been shown to activate Ras signaling (1, 4, 12, 21); this mechanism, too, could influence hypoxic transcriptional regulation. Collectively, our observations raise many more questions than they answer, but the notion that transcriptional signaling is associated with an oxidant threat to transcriptional regulatory sequences clearly has far-reaching implications for aging, somatic mutation, and a host of important pathologies.

ACKNOWLEDGMENTS

The authors thank Drs. Mykhaylo V. Ruchko and Glenn L. Wilson for helpful and provocative discussions concerning this work.

GRANTS

This work was supported in part by grants from the National Heart, Lung, and Blood Institute (RO1 HL-058234, RO1 HL-113614).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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