Oxidants in signal transduction: impact on DNA integrity and gene expression

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ABSTRACT Physiological stimuli using reactive oxygen species (ROS) as second messengers caused nucleotide-specific base modifications in the hypoxic response element of the VEGF gene in lung vascular cells, with the 3′ guanine of the HIF-1 DNA recognition sequence uniformly targeted. Modeling this effect by replacing the targeted guanine with an abasic site increased incorporation of HIF-1 and the bi-functional DNA repair enzyme and transcriptional coactivator, Ref-1/Ape1, into the transcriptional complex and engendered more robust reporter gene expression. Oxidants generated in the context of physiological signaling thus affect nuclear DNA integrity and may facilitate gene expression by optimizing DNA-protein interactions.—Ziel, K. A., Grishko, V., Campbell, C. C., Breit, J. F., Wilson, G. L., Gillespie, M. N. Oxidants in signal transduction: impact on DNA integrity and gene expression. FASEB J. 19, 387–394 (2005)

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Traditional concepts hold that nuclear DNA is relatively insensitive to damage by reactive oxygen species (ROS) compared with the mitochondrial genome. Indeed, when a variety of transformed and primary cells are exposed to xanthine or glucose oxidases, or other ROS generating systems or treated with redox cycling agents or inhibitors of the electron transport chain, mitochondrial DNA integrity is severely impaired whereas baseline density of oxidative lesions in the nuclear genome rises only slightly, if at all (1–5). Several recent studies have shown that overexpression of mitochondrially targeted DNA repair enzymes protects cells against oxidant-mediated death, which suggests that mitochondrial DNA damage may serve a sentinel function by triggering death pathways before the oxidant burden rises to a point that threatens nuclear genomic integrity (6–8).

In view of the relative insensitivity of nuclear DNA to oxidative stress, we were surprised to find in cultured pulmonary artery endothelial cells (PAECs) that hypoxia, one of numerous physiological stimuli known to use ROS as second messengers (9–13), failed to increase lesion density in the damage-prone mitochondrial genome, but instead caused oxidative base modifications in the nuclear VEGF gene (14). The base modifications in the VEGF gene were conspicuous because they occurred over the same time frame as VEGF mRNA accumulation and were nucleotide specific; that is, they were frequent in the 3′ guanine of the DNA recognition sequence for HIF-1, a key transcription factor governing VEGF expression. Given the recognition that oxidative modifications in nuclear DNA contribute to genomic instability of cells and are recognized as important for “spontaneous” carcinogenesis, aging, and the pathogenesis of several age-related neurodegenerative diseases (15–18), the observation that the VEGF gene was targeted by ROS generated in the context of hypoxic signaling prompted many questions.

A significant issue pertains to whether the unusual oxidative modifications to the VEGF gene associated with hypoxia occur in response to other physiological stimuli. Hypoxia is believed to promote mitochondrial ROS production (10, 11, 19), whereas membrane-anchored NADPH oxidase or oxidoreductases generate ROS in response to receptor-mediated agonists (13, 20). Reactive oxygen species derived from these latter sources might not have access to the nuclear compartment in a manner that mimics ROS generated in response to hypoxia. We also wondered whether the effect of hypoxia on VEGF integrity would be evident in other vascular cell types. Finally, and most important, given the effect of DNA base oxidation products on local sequence topography and flexibility, the introduction of such modifications in regulatory sequences might affect DNA-protein interactions and attendant gene expression. We therefore conducted experiments to provide proof of concept that the introduction of nucleotide-specific base modifications in response to hypoxia contributes to regulation of gene expression.

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MATERIALS AND METHODS

Rat main pulmonary artery endothelial and smooth muscle cell cultures

As described (21, 22), main pulmonary arteries were isolated from 300 g Sprague-Dawley rats killed with an overdose of Nembutal. Isolated arteries were opened and the intimal lining was scraped with a scalpel. The harvested vessels were then placed into T25 Corning flask in F12 Nutrient Mixture and Dulbecco’s modified Eagle’s medium (DMEM) mixture 1:1 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). Culture medium was changed once a week; after reaching confluence, the cells were harvested using a 0.05% solution of trypsin (Gibco BRL) and passed up to 15 times. The endothelial cell phenotype was confirmed by acetylated LDL uptake, factor VIII immunocytochemical staining, and a lack of immunostaining with smooth muscle cell α-actin antibodies (Sigma, St. Louis, MO, USA).

Denuded main pulmonary artery specimens were placed in the same media described above for isolation and culture of endothelial cells. After 3 days under standard incubator conditions the arterial specimens were removed and cells that had migrated from the explants were grown to confluence. Culture medium was changed every 3 days. Cells were harvested using a 0.05% solution of trypsin (Gibco BRL) and passed 3–12 were used for all experiments. Smooth muscle phenotype was confirmed by the presence of smooth muscle-specific α-actin (Sigma), detected by immunofluorescence microscopy as described above.

Hypoxic exposure

Endothelial and smooth muscle cells were seeded in 100 mm plates and permitted to grow until confluent. The plates were secured in Billups-Rothburg chambers, purged with gas mixtures containing 21% or 2% O2, 5% CO2, and the balance in N2, and placed in a water-jacketed incubator (Forma Scientific, Inc., Marietta, OH, USA). Periods of exposure to these environments ranged from 6 to 48 h. Partial pressures of oxygen in the two treatment groups, determined in culture environments, ranged from 6 to 48 h. Partial pressures of oxygen in the two treatment groups, determined in culture environments, ranged from 6 to 48 h.

Ligation-mediated PCR

Ligation-mediated PCR performed as described previously (4) was used to evaluate oxidative lesions at single nucleotide resolution in a ~100bp sequence within hypoxic response element of the VEGF promoter encompassing the AP-1 and HIF-1 response element. DNA from control and oxidant-treated cells was isolated, in some instances treated with alkali, then denatured to produce single-stranded DNA with terminal 5’ phosphate groups. After annealing a primer (Table 1) to the known 3' sequence, extension was performed to yield double-stranded DNA fragments whose lengths are determined by the site of cleavage; i.e., the site of the lesion. A linker (Supplement, Table 1) was then ligated to the blunt-end duplex DNA, and DNA was amplified by multiple cycles of PCR using a nested primer and another primer complementary to the linker sequence. Subsequently, the reaction mixture was run on a sequencing gel and electroblotted to a nylon membrane. The membrane was then hybridized with a single-strand probe and analyzed by DNA affinity precipitation (DNAP) analyses

DNAP analyses were performed as described by Ebert and Bunn (23). Ten pmol of biotin-labeled 64-mer oligonucleotides, one of which was a wild-type sequence corresponding to a minimal functionally active segment of the hypoxic response element of the PAEC VEGF gene and the other incorporating tetrahydrofuran at the hypoxia-modified guanine (see Fig. 2A), were incubated with 125 μg of Dynabeads M-280 streptavidin (10 mg/mL) (Dynal A.S., Oslo, Norway) for 20 min at 25°C. Biotinylated oligonucleotides bound to the streptavidin-conjugated beads were retrieved using a Magnetic Particle Concentrator (Dynal A.S.). Bead-associated oligonucleotides were washed twice with Tris-EDTA and once with buffer D (25 mM Tris-HCL (pH 7.5), 0.2 mM EDTA, 0.1M KCl, and 20% glycerol) freshly supplemented with dithiothreitol and Na3VO4 for 4 h at 4°C. The lysate was centrifuged at 15,000 × g for 30 min at 4°C, divided into 25 μl aliquots, flash frozen in liquid N2, and stored at −80°C. Nuclear protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

TABLE 1. Primers used in ligation-mediated PCR analyses of the VEGF promoter coding strand

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PhosphorImager. Lesions within the 213 bp sequence of interest were indicated by increased hybridization matched to a specific nucleotide by comparison to a Maxim-Gilbert sequence ladder. A spatial map of lesion density was constructed by expressing normalized hybridization intensities at each damaged nucleotide in the ~60 bp sequence encompassing the AP1 and HIF-1 response elements.

Nuclear protein isolation

Rat PAECs exposed to normoxic (21% O2) or hypoxic (2% O2) conditions for 3 h were washed and scraped into cold 1x PBS. Cells were centrifuged at 2000 × g for 10 min at 4°C. Supernatant was removed and the pellet was washed with 5 packed cell volumes (PCV) of buffer A (10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2, 10 mM KCl supplemented with 1M DTT, 0.2M PMSF, leupeptin (1 mg/mL), aprotinin (1 mg/mL) pepstatin (mg/mL), and 0.5M Na3VO4, resuspended in 4 PCV of buffer A, and incubated on ice for 10 min. The cell suspension was homogenized and nuclei were pelleted by centrifugation at 10,000 × g for 10 min at 4°C, then resuspended in 3 PCV of buffer C (20 mM Tris-HCl (pH 7.5), 0.42 M KCl, 1.5 mM MgCl2, 20% glycerol) and rotated for 30 min at 4°C. The suspension was centrifuged at 15,000 × g for 30 min at 4°C and dialyzed against three changes of buffer D (15 mM Tris-HCL (pH 7.5), 0.2 mM EDTA, 0.1M KCl, and 20% glycerol) freshly supplemented with dithiothreitol and Na3VO4 for 4 h at 4°C. The lysate was centrifuged at 15,000 × g for 10 min at 4°C, divided into 25 μl aliquots, flash frozen in liquid N2, and stored at −80°C. Nuclear protein concentration was determined using the Bradford method.

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within the coding strand of an mediated PCR analysis of nucleotide-specific modifications of the hypoxic response element of the VEGF promoter in rat cultured pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs) cultured in normoxia (N) or hypoxia (H) for 3 h. Also shown are ligation-mediated PCR analyses of the same sequence in PAECs challenged for 1 h with PDGF (P: 50 ng/mL), angiotensin II (A, 1 nM), or thrombin (T: 1 U/mL). Top: “—” and “+” refer to untreated DNA and DNA treated with alkali, respectively, to reveal abasic sites. A Maxam-Gilbert sequence ladder is shown at the far left, where the letters G, A, T, and C correspond to nucleotide bases guanine, adenine, thymine, and cytosine, respectively. Damage at a given nucleotide is indicated by an increase in hybridization intensity. Note prominent increases in hybridization intensity at multiple nucleotides in DNA from hypoxia-exposed cells and in cells treated with each of the 3 receptor-mediated agonists. Arrow at right denotes 3′ guanine within the HIF-1 DNA recognition sequence, which is modified in response to all of the stimuli tested in PAECs and, in PASMCs, by hypoxia. B) “Map” of alkali-detectable modifications evoked by hypoxia, PDGF, angiotensin II, and thrombin in the coding strand of the hypoxic response element of the VEGF promoter in PAECs. Position of bars reflects damage to specific nucleotides, designated by A, T, G, and C; height of bars indicates relative prevalence of damage as determined by hybridization intensities normalized to a scale of 1–6, where 1 corresponds to the least and 6 corresponds to the most intense hybridization. Each bar reflects the mean of at least 4 experiments. C) Top: map of alkali-detectable modifications evoked by hypoxia in the coding strand of the hypoxic response element of the VEGF promoter in PASMCs. HIF-1 and AP1 DNA recognition sequences are underlined, as is a palindromic CACA sequence believed to be involved in transcriptional activation. Bottom: ligation-mediated PCR analyses were performed on PASMCs cultured in hypoxia for 3–48 h. Bottom: the “percentage repair” at specific nucleotides, designated by a letter and position number corresponding to the lesion map presented in the top panel, was calculated using the 3 h time point as 0% repair according to the following formula:

\[
\text{Percentage repair} = \frac{\text{hybridization intensity at 3 h} - \text{hybridization intensity at the indicated time}}{\text{hybridization intensity at 3 h}} \times 100.
\]

each point reflects the mean of at least 4 experiments.

SDS-PAGE and Western analyses

To identify specific proteins isolated by DNA affinity precipitation, SDS-PAGE and Western analyses were used. Samples were suspended in 2× SDS-sample buffer, boiled for 2 min, and loaded onto a precast 4–15% polyacrylamide gradient Tris-HCl gel (Bio-Rad). Proteins were separated at 100 V for 1.5 h and transferred to a nitrocellulose membrane at 250 mA for 45 min using a Protein Transfer Unit (Bio-Rad). The membrane was removed from the transfer unit and subjected to Western blot analysis. First, the membrane was blocked with Blotto (5% nonfat dry milk in 1× PBS with 0.05% Tween) for 1 h. Blocking solution was removed, and 1° antibody was diluted in 5 mL Blotto and applied to the nitrocellulose membrane. Antibody concentrations and incubation conditions varied depending on the specific requirements of each antibody per the manufacturer’s recommendation: HIF-1α mouse monoclonal antibody (ab1: Novus-Biologicals, Littleton, CO, USA) was diluted 1:500 and incubated overnight at 4°C. Ref-1/APE1 mouse monoclonal antibody (NB 100-116: Novus-Biologicals) was diluted to 2 μg/mL in 5 mL Blotto and incubated for 1 h at 25°C. ATF/CREB mouse monoclonal antibody (25C10G: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted 1:100 in 5 mL Blotto and incubated at 25°C for 1.5 h. p300 mouse monoclonal antibody (NM11: NeoMarkers, Inc., Fremont, CA, USA) was diluted to 1 μg/mL in 5 mL of Blotto and incubated at 25°C for 2 h. For all of the above antibodies, goat anti-mouse HPR-conjugated 2° antibody (Bio-Rad) was diluted 1:5000 in Blotto and membranes were incubated at 25°C for 45 min. Antibody was removed and membranes were washed 3× 5 min in 1× PBS + 0.05% Tween and 1× 5 min in 1× PBS. Proteins were visualized with Luminol (Santa Cruz Biotechnology, Inc.) enhanced chemiluminescence detection system using Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, England).

Transient transfection

Rat PAECs were transfected with constructs encompassing either the wild-type or abasically modified 64-mer sequences used in the above-described DNAP analyses and a luciferase reporter gene. Because the presence of the abasic site precluded bacterial propagation of the plasmid, each experiment required preparation of new vector whose sequence was routinely verified. In brief, wild-type and abasically modified 64-mer sequences of the hypoxic response element of the rat VEGF gene were ligated into the luciferase reporter plasmid.
pGL3 (Promega, Madison WI, USA). Two μg of the circular plasmid was digested with SacI and SmaI restriction enzymes, which removed a 17 bp sequence. The restricted plasmid was run on a 1.5% agarose gel for 1.5 h at 100V, stained with ethidium bromide, and visualized using UV radiation. Visible bands were excised, placed in preweighed microcentrifuge tubes, and the restricted plasmid was isolated and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). After purification, a 65 bp insert of the VEGF promoter (20 ng) was ligated into the restricted, purified plasmid (500 ng) using T4 ligase (Promega). The ligation mixture was incubated for 18 h at 4°C, subjected to agarose gel electrophoresis, stained with ethidium bromide, then visualized with UV light. Ligated plasmids were excised from the gel, placed in microcentrifuge tubes, and purified using QIAquick Gel Extraction Kit (Qiagen). For transfections, PAECs were seeded at a density of 1.04 × 10^5/well in a 12-well (4.9 cm²/well) tissue culture dish (Costar, Corning Inc., Corning, NY, USA). Transfections were carried out with 300 ng of ligated plasmid using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s protocol. PAECs were incubated under normal conditions with the transfection complexes for 18 h, then the media was changed and cells were cultured in normoxia or hypoxia for 24 h. Luciferase activity was determined using a luminometer (Analytical Luminescence Laboratory, BD PharMingen, San Diego, CA, USA).

RESULTS

Primary cultures of PAECs were exposed to hypoxia (1% O₂) for 3 h or challenged for 1 h with platelet-derived growth factor (PDGF; 50 ng/mL), angiotensin II (1 μM), or thrombin (1 U/mL). The latter three stimuli exert their effects on PAECs via specific receptors that promote ROS generation from membrane-anchored enzymes (13). Ligation-mediated polymerase chain reaction (LMPCR) analysis was used to evaluate the relative frequency of oxidative modifications to DNA at single nucleotide resolution in a ~65 bp sequence of the VEGF promoter encompassing the hypoxic response element. Representative autoradiograms depicting the effects of 3 h hypoxic culture or treatment with the three receptor-mediated agonists on integrity of the hypoxic response element in the VEGF promoter in PAECs are shown in Fig. 1A. Increased hybridization intensities are indicative of high-frequency modifications; the specific nucleotide modified is determined by relating the positions of the hybridization bands to the Maxam-Gilbert sequence gel shown to the left of the autoradiograms. It is apparent that all of the stimuli promote modifications at multiple nucleotides, that purines are often modified, and that certain sites are more commonly targeted. In addition, treatment of the DNA with alkali, which causes single-strand breaks at sites of oxidant-mediated base loss, termed “abasic sites,” enhanced hybridization intensities regardless of the initiating stimulus. Finally, and consistent with our previous report (14), we found that modifications evoked by the selected stimuli were abrogated by the antioxidant DMTU (data not shown), providing additional evidence for an ROS-dependent mechanism. Frequency maps of alkali-detectable modifications at specific nucleotides evoked by hypoxia, thrombin, angiotensin II, and PDGF in PAECs are shown in Fig. 1B. A number of nucleotides were modified at relatively high frequency by the selected stimuli, but the guanine residing at the extreme 3' end of the HIF-1 DNA recognition sequence was uniformly targeted (arrow, Fig. 1A). These findings indicate that, in PAECs, integrity of the nuclear VEGF gene is threatened not only by ROS generated in hypoxic signaling, but also by ROS generated from membrane-anchored sources in response to several receptor mediated agonists.

Another question to arise concerning the effect of hypoxia on integrity of the VEGF promoter is whether this effect occurred in cells other than PAECs. Accordingly, cultured pulmonary artery smooth muscle cells (PASMCs) were exposed to hypoxia and LMPCR was used to assess modifications at single nucleotide resolution. A representative autoradiogram derived from PASMCs exposed to hypoxia for 3 h is shown in Fig. 1A and a frequency map of modifications in the VEGF hypoxic response element is shown in Fig. 1C. The pattern of hypoxia-induced modifications in PASMCs differed somewhat from that in PAECs. The density of

Figure 2. Predicted curvature (red line) and predicted bendability (blue line) of the hypoxic response element of the rat PAEC VEGF gene. Note that terminal guanines of the HIF-1 DNA recognition sequence (underlined) and guanine oxidatively modified by hypoxia, PDGF, angiotensin II, and thrombin comprise the stiffest region of the sequence.
low-frequency alterations in the VEGF promoter in PASMCs was greater than in PAECs; unlike the latter cell type, where modifications were largely restricted to purines, both purines and pyrimidines were modified in hypoxic PASMCs. Most important, however, the 3’ guanine of the HIF-1 DNA recognition sequence was again one of the most frequently modified nucleotides.

Time-dependent effects of hypoxia on the hypoxic response element in the VEGF gene of PASMCs were assessed using LMPCR analyses. Peak density of alkali-detectable modifications occurred at 3 h of hypoxia; Fig. 1C shows the percentage hybridization intensities at selected nucleotides as a function of the duration of hypoxic exposure. Despite the continued hypoxic exposure, base modifications at some nucleotides were removed relatively quickly while others persisted. For example, the guanine in the HIF-1 DNA recognition sequence along with an adenine located in a functionally relevant CACA sequence, believed to be important for transcriptional activation but not involved with transcription factor binding (24), were repaired most rapidly, whereas modifications in other nucleotides with no known functions remained for the duration of hypoxic exposure. Because DNA base oxidation products are known to cause profound changes in local sequence topology and flexibility (25–28), such nucleotide-specific, time-dependent removal of oxidative modifications suggests a model in which oxidant stress associated with physiological signaling promotes complex changes in local sequence topology and flexibility of the hypoxic response element.

If the introduction of ROS-mediated base oxidation products on local sequence topography and flexibility, the introduction of such modifications in regulatory sequences, as shown by the observations presented above, might affect DNA-protein interactions and attendant gene expression. To provide proof-of-concept for this idea, initial experiments used DNA affinity precipitation analyses, performed as described (23), to assess the composition of the hypoxia-inducible transcriptional complex forming on a wild-type hypoxic response element from the VEGF gene and on a corresponding sequence harboring model abasic sites introduced at the hypoxia-modified guanine (Fig. 3A). We selected an abasic site to mimic the effect of hypoxia because of the ability of alkali treatment of the DNA to enhance lesion intensity (see Fig. 1A) and because abasic sites are key intermediates in the base excision repair pathway that removes oxidative DNA damage (32, 33). In the first modified oligonucleotide, tetrahydrofuran was introduced at the hypoxia-modified guanine to create a stable abasic site. Wild-type and abasically modified

![Figure 3. A) Wild-type (WT) sequence of the hypoxic response element of the VEGF gene and a similar sequence harboring either a tetrahydrofuran or a natural abasic site at the guanine shown by ligation-mediated PCR analyses to be modified by hypoxia and other physiological stimuli using ROS as second messengers. B) Western blot analysis, representative of 6 experiments, of proteins associating with the hypoxic response element of the VEGF promoter as recovered by DNA affinity precipitation using the WT and abasically modified (Abasic) oligonucleotide probes shown in panel A. Note increased abundance of HIF-1 and Ref-1/Ape1 associated with the abasically modified oligonucleotide probe compared with the WT probe.](image)
oligouonucleotide probes were incubated with nuclear protein extracts derived from PAECs cultured in normoxia or hypoxia for 3 h and Western blot analyses were used to identify probe-associated proteins. As shown in Fig. 3B, p500, ATF, CREB, Ref-1/Ape1, and, when nuclear extract from hypoxic cells was used, HIF-1 associated with the wild-type oligonucleotide probe. The abasically modified oligonucleotide probe associated with these same proteins but, remarkably, the abundances of HIF-1 and Ref-1/Ape1 were noticeably increased. A second oligonucleotide probe incorporated uracil at the hypoxia-modified guanine. The probe was then treated with uracil glycosylase, which removed the uracil base, engendering formation of a natural, but relatively unstable, abasic site. DNA affinity precipitation analyses indicated that the uracil glycosylase-induced formation of a natural abasic site at the hypoxia-modified guanine was associated with increased abundances of HIF-1 and Ref-1/Ape1 compared with the transcriptional complex forming on the wild-type oligonucleotide probe (data not shown).

HIF-1 was initially described as the key transcription factor driving hypoxia-induced gene expression, but is now understood to play a central role in enhancing gene expression in response to receptor-dependent stimuli as well, including thrombin and PDGF (34–36). The increase in HIF-1 incorporated in the hypoxia-inducible transcriptional complex forming on the abasically modified oligonucleotide probes corresponding to the hypoxic response element could thus be expected to positively affect gene expression. We addressed this possibility by transfecting PAECs with constructs composed of a luciferase reporter gene linked to either a wild-type hypoxic response element or a hypoxic response element incorporating tetrahydrofuran at the hypoxia-modified guanine to mimic a stable abasic site. Transfected PAECs were cultured in normoxia or hypoxia for 24 h, then luciferase gene expression was assessed. As shown in Fig. 4, whereas hypoxia increased expression of the reporter gene driven by the wild-type hypoxic response element by 180 ± 15%, induction was 370 ± 65% above normoxic controls in PAECs transfected with the abasically modified hypoxic response element.

DISCUSSION

These results show that several different stimuli using ROS as second messengers promote oxidative modifications in the promoter of the VEGF gene in two vascular cell types. Along with numerous cytosolic proteins, transcription factors, membrane ion channels, and transporters, nuclear DNA thus appears to be a target of ROS generated in physiological signaling. Although the nuclear genome is far less sensitive to oxidative damage than mitochondrial DNA, there is nevertheless a detectable level of oxidative modifications in nuclear genes, which probably reflects the balance between ROS production and the activity of specific DNA repair mechanisms (37–40). The low, steady-state levels of oxidative modifications in nuclear DNA are believed to contribute to genomic instability of cells and are recognized as important for “spontaneous” carcinogenesis, aging, and the pathogenesis of several age-related neurodegenerative diseases (15–18, 41). In an extension of this paradigm, Lu and co-workers reported that aging of the human brain is accompanied by marked increases in oxidative damage to genes involved in learning, memory, and neuronal survival (42). The oxidative damage was most prominent in promoter sequences and associated with reduced efficiency of base excision repair. Traditional concepts hold that the nuclear DNA damaging ROS are by-products of the mitochondrial electron transport chain. Recently, however, this well-entrenched idea has been challenged by findings that cells depleted of mitochondrial DNA or the antioxidant glutathione or challenged with pharmacologically induced increases in mitochondrial ROS production fail to exhibit changes in the density of nuclear DNA damage (5), suggesting there is another, more important source of ROS contributing to the steady-state level of nuclear DNA modifications. The observations reported herein suggest that ROS generated as part of signal transduction could be important determinants of nuclear DNA lesion density.

Experiments to model the effect of hypoxia and the receptor-mediated agonists by introducing abasic sites at the targeted 3’ guanine in the HIF-1 DNA recognition sequence provide proof-of-concept for the hypothesis that such modifications contribute to regulation of gene expression. Incorporation of model abasic sites altered the composition of the hypoxia-inducible transcriptional complex, increasing the abundance of the
key transcription factor HIF-1 and the ubiquitous, bifunctional regulatory protein Ref-1/Ape1. In addition, expression of a reporter gene driven by a hypoxic response element harboring an abasic site at the hypoxia-modified guanine is ~2-fold greater than that driven by a wild-type promoter sequence. The potential involvement of Ref-1/Ape1 in hypoxic gene expression deserves special consideration. Ref-1/Ape1 is widely appreciated as an important co-transcriptional protein; its N-terminal domain interacts directly with at least nine different transcriptional activating proteins, including HIF-1 (33), and we recently found that it is essential for formation of the transcriptional complex on the wild-type hypoxic response element of the VEGF gene (43). Since the C-terminal domain of Ref-1/Ape1 recognizes and binds abasic sites in duplex DNA, it seems likely that oxidative base modifications in the HIF-1 DNA recognition sequence serve as a substrate for Ape1 binding, which then positions the Ref-1 domain to function as a scaffold for optimal assembly and activation of the transcriptional complex.

The unusual sensitivities of the guanines in HIF-1 DNA recognition sequence to oxidative modification has been noted, but not in association with ROS used in physiological signaling. Rodriguez and co-workers treated isolated DNA with an ROS generating mixture of copper and hydrogen peroxide and, using LM-PCR, found that the HIF-1 DNA recognition sequence in the human PGK1 gene was a “hot spot” for damage (44). The reason that the terminal guanine in the HIF-1 DNA recognition sequence is prone to oxidative modifications may relate to the fact that iron preferentially localizes within the sequence R-G-T-R compared with other sequences (45, 46). The modified guanine is in the immediate vicinity of an R-G-T-R sequence and is the terminal guanine in a triplet repeat. Guanine repeats have been termed the “π-way” through which electrons tunnel to oxidatively modify the terminal guanine in the sequence (47, 48). Thus it is reasonable to suspect that the 3′ guanine within the HIF-1 DNA recognition sequence is targeted by hydroxyl radical generated by Fenton chemistry within the R-G-T-R sequence in close, 5′ proximity to the modified guanine. R-G-T-R sequences are common in transcription factor binding motifs (45), suggesting that the oxidative base modifications in and around the HIF-1 DNA recognition sequence noted here may occur in other transcription factor recognition sequences.

In summary, our findings demonstrate that a nuclear gene, traditionally regarded as resistant to ROS-mediated damage, is modified in a nucleotide-specific manner by oxidants used as second messengers in physiological signaling. Because the modifications are prominent within a functionally significant sequence of the VEGF gene and since introduction of an abasic site to model the ROS-mediated effect promotes changes in the transcriptional complex and attendant increases in reporter gene expression, we propose that ROS-dependent, nucleotide-specific modifications may comprise a new mechanism contributing to regulation of gene expression. In light of the probable role of oxidative nuclear DNA modifications in cancer and other disorders, the present findings raise the intriguing possibility that, with the passage of time, ROS required for signaling threaten genomic integrity, thus ensuring that the longevity of each cell is limited by the very processes required for its life.

REFERENCES