Free Radical Biology and Medicine 96 (2016) 78–88

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Regulation of mitochondrial genome replication by hypoxia: The role of DNA oxidation in D-loop region

Viktor M. Pastukh, Olena M. Gorodnya, Mark N. Gillespie, Mykhaylo V. Ruchko *

Department of Pharmacology and Center for Lung Biology, University of South Alabama College of Medicine, Mobile, AL 36688, USA

ARTICLE INFO

Article history:
Received 21 November 2015
Received in revised form
18 March 2016
Accepted 14 April 2016

Keywords:
Hypoxia
Reactive oxygen species
Oxidative DNA modifications
D-loop
Mitochondrial DNA replication
TFAM binding
Ogg1 overexpression

ABSTRACT

Mitochondria of mammalian cells contain multiple copies of mitochondrial (mt) DNA. Although mtDNA copy number can fluctuate dramatically depending on physiological and pathophysiologic conditions, the mechanisms regulating mitochondrial genome replication remain obscure. Hypoxia, like many other physiologic stimuli that promote growth, cell proliferation and mitochondrial biogenesis, uses reactive oxygen species as signaling molecules. Emerging evidence suggests that hypoxia-induced transcription of nuclear genes requires controlled DNA damage and repair in specific sequences in the promoter regions. Whether similar mechanisms are operative in mitochondria is unknown. Here we test the hypothesis that controlled oxidative DNA damage and repair in the D-loop region of the mitochondrial genome are required for mitochondrial DNA replication and transcription in hypoxia. We found that hypoxia had little impact on expression of mitochondrial proteins in pulmonary artery endothelial cells, but elevated mtDNA content. The increase in mtDNA copy number was accompanied by oxidative modifications in the D-loop region of the mitochondrial genome. To investigate the role of this sequence-specific oxidation of mitochondrial genome in mtDNA replication, we overexpressed mitochondria-targeted 8-oxoguanine glycosylase Ogg1 in rat pulmonary artery endothelial cells, enhancing the mtDNA repair capacity of transfected cells. Overexpression of Ogg1 resulted in suppression of hypoxia-induced mtDNA oxidation in the D-loop region and attenuation of hypoxia-induced mtDNA replication. Ogg1 overexpression also reduced binding of mitochondrial transcription factor A (TFAM) to both regulatory and coding regions of the mitochondrial genome without altering total abundance of TFAM in either control or hypoxic cells. These observations suggest that oxidative DNA modifications in the D-loop region during hypoxia are important for increased TFAM binding and ensuing replication of the mitochondrial genome.

1. Introduction

Reactive oxygen species (ROS) from mitochondria and other endogenous and exogenous sources constantly generated in living cells have the potential to damage lipids, proteins, RNA and DNA [1]. DNA damage, in particular, may trigger cell death pathways or lead to mutations [2]. Pathogenic oxidant stress is directed not only at the nuclear genome; oxidative damage and resulting mutations in the mitochondrial DNA also are linked to a range of human disorders, including aging, cancer, neurodegenerative and cardiovascular diseases [3,4].

Traditional concepts hold that maintenance of DNA integrity is indispensable to normal cellular physiology; damage must be repaired, or cells die or harbor potentially deleterious mutations. However, there is emerging evidence that, at least for nuclear genes, controlled DNA damage and repair may be necessary for normal transcriptional regulation. For example, *in vitro* studies on the p50 subunit of the NF-κB transcription factor binding to the NF-κB promoter showed that oxidation of guanine at sites critical for protein recognition increases p50 binding affinity [5]. In MCF-7 cells, activation of estrogen responsive elements in selected genes leads to transient, oxygen radical-mediated DNA strand breaks that appear to be required for long-range changes in DNA topography and increased mRNA expression [6,7]. We have shown

Abbreviations: ANOVA, analysis of variance; ATP6, ATP synthase subunit 6; BER, base excision repair; ChIP, chromatin immunoprecipitation; Cox2, cytochrome c oxidase subunit 2; DIG, digoxigenin; Fpg, formamidopyrimidine DNA glycosylase; mtDNA, mitochondrial DNA; ND4, NADH dehydrogenase subunit 4; Ogg1, 8-oxoguanine glycosylase; PAECs, pulmonary artery endothelial cells; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SE, standard error; TE, Tris-EDTA; TFAM, mitochondrial transcription factor A; VEGF, vascular endothelial growth factor

* Correspondence to: University of South Alabama, Department of Pharmacology, 5851 USA Dr. N., MSB 3370, Mobile, AL 36688, USA.
E-mail addresses: vpastukh@southalabama.edu (V.M. Pastukh), gorodnya@southalabama.edu (O.M. Gorodnya), mgillesp@southalabama.edu (M.N. Gillespie), mruchko@southalabama.edu (M.V. Ruchko).

http://dx.doi.org/10.1016/j.freeradbiomed.2016.04.011
0891-5849/

previously that a similar pathway is operative in pulmonary artery endothelial cells (PAECs) where hypoxia, like other physiologic signals using reactive oxygen species as second messengers [8,9], causes ROS-dependent modifications at specific bases within hypoxia-response elements of hypoxia-inducible genes [10–12]. The oxidative lesions associated with hypoxic signaling are temporally related to mRNA accumulation [11] and restricted to hypoxia-response elements associated with transcriptionally-active nucleosomes [13]. Mimicking the effect of hypoxia by introducing model oxidative base modifications in the hypoxia-response element of the VEGF promoter leads to enhanced sequence flexibility, altered transcription factor binding and more robust reporter gene expression [10,14]. Collectively, these findings support the concept that controlled, ROS-mediated nuclear DNA damage and repair are associated with normal physiologic signaling and function to alter the topology and flexibility of key promoter sequences thereby facilitating regulatory protein binding and productive transcription [7,15]. These observations raise an intriguing question relative to the mitochondrial genome: could a similar model for ROS-dependent transcriptional activation be operative in mitochondria?

In mammalian cells, each of the hundreds-to-thousands of mitochondria per cell harbors 2–10 copies of mitochondrial DNA [16]. Increases in the cellular contents of mtDNA can be stimulated by a variety of metabolic and/or pathophysiologic stresses. For example, increased mtDNA content has been reported in aging tissues [17–19], some cancers [20], in cells treated with lipopolysaccharide [21], and in cells treated with non-lethal concentrations of hydrogen peroxide [22]. In many cell types, including lung cells, both hypoxia and hyperoxia can also stimulate an increase in mtDNA content [23–27]. Importantly, a feature common to all of these diverse conditions is increased oxidant stress. However, the mechanism by which oxidant stress stimulates mtDNA replication remains unknown.

As discussed subsequently, multiple lines of indirect evidence suggest that “DNA damage and repair pathway” believed to govern nuclear gene expression also functions to regulate mtDNA replication. In many cell types, oxidant stress leads to upregulated expression of the key transcription factor driving mitochondrial gene expression and mtDNA replication, mitochondrial transcription factor A (TFAM) [28]. TFAM, being the main structural protein of the nucleoid, plays a significant role not only in mitochondrial genome transcription and replication, but also in packaging and repair of the mtDNA [29]. TFAM initiates mitochondrial transcription by binding to a non-coding regulatory sequence known as the D-loop region, which contains light-strand and heavy-strand promoter sequences. While the fine mechanism of mtDNA replication remains controversial, both existing models of this process hold that mtDNA synthesis requires extension of RNA primers, the generation of which is mediated by transcription machinery [30]. Thus, the TFAM-mediated processes of mtDNA transcription and replication are tightly and inextricably linked [31,32].

The D-loop region is known to be exquisitely sensitive to oxidant stress. In this regard, while mtDNA is about 30-fold more sensitive to ROS-mediated damage than the nuclear genome [33], the few studies focusing on the D-loop region suggest that it is even more prone to oxidant attack than the coding portion of the mitochondrial genome [34,35]. It has also been reported that incorporation of oxidative base damage products into model oligonucleotides enhances DNA binding affinity for TFAM [36].

Previously, we have successfully shown that hypoxia induces ROS production in rat pulmonary artery endothelial cell culture [8,9] and that mitochondria are involved in this process [8,13,37]. Against this background, the present study tested the hypothesis that controlled oxidative DNA damage and repair in the D-loop region of the mitochondrial genome are required for mtDNA replication and transcription in hypoxia. To address this issue and to decrease hypoxia-induced mtDNA oxidative damage, we used our previously published strategy – targeting human DNA glycosylase Ogg1 (hOgg1), an enzyme executing the first step in base excision repair, to mitochondria of rat pulmonary artery endothelial cells [38]. Oxidative stress can result in a variety of mtDNA lesions, including single-strand breaks, abasic sites and oxidized DNA bases, among which guanine is the base most susceptible to oxidation [39]. Mitochondria employ multiple DNA repair mechanisms to repair ROS-induced damage, but base excision repair (BER) is the primary pathway used to remove oxidatively-modified DNA bases [39,40]. Several DNA glycosylases responsible for recognition and removal of base lesions during the first step in the BER pathway have been identified in mitochondria, including Ogg1, NEIL1 and NEIL2, MutY homolog MYH, Endo III homolog NTH1, and uracil DNA glycosylase UNG1 [40]. Ogg1 and NEIL enzymes have overlapping specificity to 8-oxoguanine; nevertheless, Ogg1 remains the primary enzyme for the repair of oxidized purines [40,41]. In our previous work we showed that cells deficient in Ogg1 demonstrated increased mtDNA damage and oxidant-mediated apoptosis, indicating that Ogg1 may be a rate-limiting enzyme in the mitochondrial BER [42]. On the other hand, overexpression of mitochondria-targeted human Ogg1 in rat PAECs and other cell types significantly enhanced DNA repair capacity and protected mtDNA from oxidant-induced damage [38,43–45]. In the present study we expected that overexpression of mitochondria-targeted hOgg1 would improve mitochondrial DNA repair and, thus, decrease hypoxia-induced oxidative damage to the mitochondrial genome. Then we analyzed replication and transcription of mtDNA in control cells and cells transfected with hOgg1 under hypoxic conditions. Abundance of TFAM, as a key mitochondrial transcription factor, and its binding to the mtDNA were also studied.

2. Materials and methods

2.1. Cell culture and treatment

Rat PAECs were harvested and cultured as described previously [42]. Control (normoxic) cells were cultured in a water-jacketed incubator purged with air+5% CO_2, while hypoxic cells were cultured for the indicated periods in an incubator purged with air, N_2, and CO_2 to create an environment consisting of 2% O_2, 5% CO_2, and 93% N_2.

2.2. Ogg1 overexpression and its assessment

We stably transfected rat PAECs with lentivirus to overexpress hOgg1 in mitochondria. Empty lentiviral vector and vector containing hOgg1 gene were prepared as described previously [38]. Transfected cells were cultured in medium containing 10 μg/ml puromycin for 72 h to exclude cells without lentiviral construct. Transfection was confirmed by real time and conventional RT-PCR analyses, and Western blot analysis as described earlier [11,38]. Briefly, for quantitative RT-PCR analysis total RNA was isolated from rat PAECs using PrepEase RNA Spin Kit (Afflymetrix, Santa Clara, CA) and quantitative real-time RT-PCR was then performed using the USB VeriQuest SYBR Green One-Step qRT-PCR Master Kit with Fluorescein (Afflymetrix, Santa Clara, CA) according to the manufacturer's protocol using sets of primers for rat and human Ogg1 listed in Table 1. Immunoblotting analysis was performed using an antibody to Ogg1 (Abcam, Cambridge, UK).

2.3. Isolation of subcellular fractions and Western immunoblot