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DIFFERENT ORGANIZATION OF BASE EXCISION REPAIR OF URACIL IN DNA IN NUCLEI AND MITOCHONDRIA AND SELECTIVE UPREGULATION OF MITOCHONDRIAL URACIL-DNA GLYCOSYLASE AFTER OXIDATIVE STRESS

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Abstract—Oxidative stress in the brain may cause neurodegeneration, possibly due to DNA damage. Oxidative base lesions in DNA are mainly repaired by base excision repair (BER). The DNA glycosylases Nei-like DNA glycosylase 1 (NEIL1), Nei-like DNA glycosylase 2 (NEIL2), mitochondrial uracil-DNA glycosylase 1 (UNG1), nuclear uracil-DNA glycosylase 2 (UNG2) and endonuclease III-like 1 protein (NTH1) collectively remove most oxidized pyrimidines, while 8-oxoguanine-DNA glycosylase 1 (OGG1) removes oxidized purines. Although uracil is the main substrate of uracil-DNA glycosylases UNG1 and UNG2, these proteins also remove the oxidized cytosine derivatives isodialuric acid, alloxan and 5-hydroxyuracil. UNG1 and UNG2 have identical catalytic domain, but different N-terminal regions required for subcellular sorting. We demonstrate that mRNA for UNG1, but not UNG2, is increased after hydrogen peroxide, indicating regulatory effects of oxidative stress on mitochondrial BER. To examine the overall organization of uracil-BER in nuclei and mitochondria, we constructed cell lines expressing EYFP (enhanced yellow fluorescent protein) fused to UNG1 or UNG2. These were used to investigate the possible presence of multiprotein BER complexes in nuclei and mitochondria. Extracts from nuclei and mitochondria were both proficient in complete uracil-BER *in vitro*. BER assays with immunoprecipitates demonstrated that UNG2-EYFP, but not UNG1-EYFP, formed complexes that carried out complete BER. Although apurinic/apyrimidinic site endonuclease 1 (APE1) is highly enriched in nuclei relative to mitochondria, it was apparently the major AP-endonuclease required for BER in both organelles. APE2 is enriched in mitochondria, but its possible role in BER remains uncertain. These results demonstrate that nuclear and mitochondrial BER processes are differently organized. Furthermore, the upregulation of mRNA for mitochondrial UNG1 after oxidative stress indicates that it may have an important role in repair of oxidized pyrimidines.

Key words: base excision repair, oxidative stress, uracil-DNA glycosylase, repair complexes, mitochondria.

Defective DNA repair is associated with neuro-developmental problems and neurodegeneration. This is well established for deficiencies in nucleotide excision repair (NER), transcription-coupled repair (TCR), and DNA damage-signaling (Krokan et al., 2004; Cleaver, 2005). Base excision repair (BER) of DNA is the major mechanism for repair of base lesions that do not cause helix distortions (Krokan et al., 1997). This pathway repairs a wide variety of oxidative lesions in purines and pyrimidines in nuclear and mitochondrial DNA (mtDNA) (Slupphaug et al., 2003). Generation of reactive oxygen species (ROS) from the mitochondrial electron-transport chain is an unavoidable by-product of aerobic respiration (Margaill et al., 2005). Therefore, mitochondrial BER of oxidative damage may be particularly important for protection of the brain, which utilizes as much as 20% of the oxygen consumed by the body, but makes up only 2% of the body weight (Dringen, 2000). Generation of ROS increases dramatically after ischemia or brain injury, and this is thought to be important for death of brain neurons after global or focal ischemia (Lipton, 1999; Margaill et al., 2005). The particular relevance of mitochondrial damage and repair in this context is illustrated by the finding that mtDNA repair deficiencies correlate with oxidative stress-induced apoptosis in neuronal cells (Harrison et al., 2005). DNA glycosylases initiate BER by releasing the damaged base, leaving an abasic site (AP-site) that is cleaved by an AP-endonuclease. In nuclei BER is completed by short patch repair in which a single nucleotide is inserted, or long patch repair in which two to eight nucleotides are inserted prior to ligation (Dogliotti et al., 2001). In mitochondria, short patch repair is apparently the major pathway (Stierum et al., 1999). Nuclei and mitochondria use largely different proteins for BER (Dianov et al., 2001; Larsen et al., 2005), but nuclear and mitochondrial isoforms of DNA glycosylases are frequently encoded by the same gene (Slupphaug et al., 1993; Nilsen et al., 1997; Takao et al., 1998; Nishioka et al., 1999).

Uracil-DNA glycosylases (UDG) UNG1 (mitochondria) and UNG2 (nuclear) are both encoded by the *UNG*-gene.

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Abbreviations: AP, apyrimidinic; APE1, apurinic/apyrimidinic site endonuclease 1; APE2, apurinic/apyrimidinic site endonuclease 2; BER, base excision repair; CMV, cytomegalovirus; DTT, dithiothreitol; EDTA, ethylendiaminetetraacetic acid; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; Hepes, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; hMYH, human MutY homologue; HRP, horseradish peroxidase; IP, immunoprecipitation; IP-dep., immuno-depleted; mtDNA, mitochondrial DNA; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PCV, packed cell volume; PHA, phytohemagglutinin; rec. UNG, recombinant UNG; ROS, reactive oxygen species; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; UDG, uracil-DNA glycosylase; UNG1, uracil-DNA glycosylase 1 (mitochondrial); UNG2, uracil-DNA glycosylase 2 (nuclear); XRCC1, X-ray cross-complementing protein 1.

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These enzymes constitute the major uracil-removing enzymes in human cells. UNG1 and UNG2 have a common catalytic domain, but different N-terminal domains required for subcellular sorting (Nilsen et al., 1997). The mitochondrial preform of UNG1 contains 35 unique N-terminal amino acids, approximately 29 of which are removed upon mitochondrial import to give the mature UNG1 (Bharati et al., 1998). Nuclear UNG2 has 44 unique N-terminal amino acids, all of which are retained after nuclear import (Nilsen et al., 1997; Akbari et al., 2004). There is no overlap between these sequences which are encoded by different exons. Uracil residues in DNA generally result from misincorporation of dUMP during replication or spontaneous deamination of cytosine. However, in B-cells uracil present in the Ig locus is generated by activation induced cytidine deaminase (AID) as a physiological intermediate in the acquired immune response.

UNG-proteins have catalytic turnover numbers that are several orders of magnitude higher than those of other DNA glycosylases. Although they are highly specific for uracil they also remove 5-flurouracil and oxidation products of cytosine (alloxan, isodialuric acid and 5-hydroxyuracil) with reasonable efficiencies (Dizdaroglu et al., 1996; An et al., 2005). Other oxidative lesions to pyrimidines and purines are removed by DNA glycosylases Nei-like DNA glycosylase 1, Nei-like DNA glycosylase 2 and 8-oxoguanine-DNA glycosylase 1 (Slupphaug et al., 2003). DNA glycosylase human MutY homologue (hMYH) removes mutagenic A-residues incorporated opposite of template 8-oxoG, a major oxidative purine lesion. Interestingly, mitochondrial hMYH is upregulated in substantia nigra of patients suffering from Parkinson's disease (Arai et al., 2006).

Focal and general hypoxia represents an important source of oxidative stress to the brain, as demonstrated in occlusion–reperfusion studies. Thus, brief middle cerebral artery occlusion induced oxidative mtDNA damage and increased levels of the principal enzymes of the mitochondrial BER pathway, including uracil DNA glycosylase, apurinic/apyrimidinic (AP) endonuclease, DNA polymerase γ, and DNA ligase in the rat (Chen et al., 2003). Furthermore, *Ung*$^{-/-}$ fibroblasts and primary cortical neurons showed increased cell death when treated with a nitric oxide donor and oxygen-glucose deprivation, respectively, when compared with cells from *Ung*$^{+/+}$ mice. In addition, *Ung*$^{-/-}$ mice displayed major increases in infarct size after focal-brain ischemia as compared with the control mice. In mice brain (Endres et al., 2004) and human cultured cells (Kyng et al., 2003) UDG activity was increased after cerebral ischemia. These studies indicate a significant role of UNG-proteins in the defense against oxidative brain damage. Recent studies also indicate that uracil may be generated in significant quantities in DNA after oxidative stress (Endres et al., 2004; An et al., 2005). The substrate specificity of UNG-proteins, together with sensitivity of the brain of *Ung*$^{-/-}$ mice to oxidative stress, point to uracil and/or the other cytosines derivatives (alloxan, isodialuric acid or 5-hydroxyuracil) as likely toxic lesions in the brain after oxidative stress.

MtDNA is associated with the inner membrane (Albring et al., 1977) and in several organisms it binds to distinct proteins, forming so-called nucleoids probably helping to anchor DNA to the inner membrane (Bogenhagen et al., 2003; Garrido et al., 2003). The mitochondrial inner membrane has high protein content and many, e.g. proteins involved in energy production, form large protein complexes (Schagger, 2001). Recently, several mitochondrial BER proteins were also reported to be associated with the inner membrane (Stuart et al., 2005), but there is no available information on direct interaction between mitochondrial BER proteins. In contrast, direct interaction between mammalian nuclear BER proteins has been reported for a large number of proteins, e.g. UNG2 and proliferating cell nuclear antigen (PCNA), and X-ray cross-complementing protein 1 (XRCC1) and DNA polymerase β, to mention a few (Fan and Wilson, 2005). Furthermore, a fraction of BER proteins apparently forms relatively stable complexes that can be isolated by biochemical fractionation or immunoprecipitation (IP) (Prasad et al., 1996; Akbari et al., 2004).

Here, we present results showing that p21 and UNG1, but not UNG2, are significantly (two- to threefold) upregulated in human cells after oxidative stress, while a number of other repair and replication associated proteins remain essentially unchanged. Furthermore, we demonstrate that nuclear and mitochondrial BER processes for removal of uracil are organized differently. A large fraction of nuclear UNG2 is present in a multiprotein complex fully competent in BER, while mitochondrial UNG1 is not found in a similar complex, but may instead be located in the inner mitochondrial membrane, as suggested (Stuart et al., 2005).

EXPERIMENTAL PROCEDURES

Chemicals and antibodies

Synthetic oligonucleotides were purchased from MedProbe (Oslo, Norway). [α-^{32}P]dTTP (3000 Ci/mmol), and [γ-^{33}P]ATP (3000 Ci/mmol) were from Amersham Biosciences GE Healthcare UK Ltd., Little Chalfont, UK). Primary antibodies against apurinic/apyrimidinic site endonuclease 1 (APE1) (mouse monoclonal, ab194), apurinic/apyrimidinic site endonuclease 2 (APE2) (rabbit polyclonal, ab13691), and serum anti-green fluorescent protein (GFP)-antibody (rabbit polyclonal, ab290), were from Abcam Ltd., Cambridge, UK. The GFP antibody recognizes and binds to enhanced yellow fluorescent protein (EYFP) and is hereafter referred to as anti-EYFP-Ab. Rabbit polyclonal antibodies against APE1, NB 100–101, were from Novus Biologicals Inc., Littleton, CO, USA. Normal IgG from non-immunized rabbits (sc-2027) was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Anti-UNG-antibody against the catalytic domain (PU101) has been described previously (Slupphaug et al., 1995).

Cell culture and cell lines

We prepared Mito-UNG1-UNG2-EYFP, UNG1-EYFP and UNG2-EYFP fusion proteins under control of a cytomegalovirus (CMV) promoter by transfecting HeLa cells with pUNG1$_{1-39}$UNG2-EYFP (Otterlei et al., 1998), pUNG1-EYFP and pUNG2-EYFP (Nilsen et al., 1997), respectively. The cells were then cultured under selection with 0.4 mg/ml genetecin (G418) in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.03% glutamine, and 0.1 mg/ml gentamicin in 5% CO_2. The UNG1/UNG2-EYFP posi-