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Ischemic injury and faulty gene transcripts in the brain

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Abstract

The brain has the highest metabolic rate of all organs and depends predominantly on oxidative metabolism as a source of energy. Oxidative metabolism generates reactive oxygen species, which can damage all cellular components, including protein, lipids and nucleic acids. The processes of DNA repair normally remove spontaneous gene damage with few errors. However, cerebral ischemia followed by reperfusion leads to elevated oxidative stress and damage to genes in brain tissue despite a functional mechanism of DNA repair. These critical events occur at the same time as the expression of immediate early genes, the products of which *trans*-activate late effector genes that are important for sustaining neuronal viability. These findings open the possibility of applying genetic tools to identify molecular mechanisms of gene repair and to derive new therapies for stroke and brain injury.

Brain injury of the ischemia—reperfusion type, which occurs in stroke and cardiac arrest, induces neuronal damage. The mechanisms of neuronal injury include decreased intracellular pH and ATP concentration and increased levels of extracellular glutamate, intracellular Ca^{2+} and reactive oxygen species (ROS). ROS are generated as byproducts of oxygen metabolism. Under physiological conditions, most ROS are generated in mitochondria, but pathological conditions can cause ROS to form in the cytoplasm as well (Box 1). Mitochondrial DNA is prone to damage¹ and mitochondria have functional repair processes². Because of a short half-life, the hydroxyl radicals generated in the mitochondria under normal conditions might not react with nuclear DNA to a significant extent. In experimental models of ischemia, ROS are produced within 30 minutes of the ischemic episode, predominantly in the penumbral region³. Recent studies show that the radical form of nitric oxide and superoxide anion might participate in nuclear gene damage in the brain (Box 1). The content of nitric oxide increases in the CNS after cerebral ischemia—reperfusion, traumatic head injury and spinal cord injury^{4–7}. The nitric oxide radical has dual effects: first, it combines with the superoxide anion to form peroxynitrite; second, it interferes with superoxide dismutase, whose antioxidant effect is reduced^{8,9}. The mechanisms by which peroxynitrite damages nuclear DNA are unclear, but could involve diffusion from the mitochondria and cytoplasm and cleavage in the nucleus to form hydroxyl radicals or singlet oxygen^{10,11}. Brain injury also results in an elevated extracellular concentration of glutamate, which activates neuronal nitric oxide synthase via

Ca^{2+} influx^{12,13}. Other routes that induce the formation of ROS have been presented in recent reviews^{14–16}. There is evidence that the cellular accumulation of nitric oxide can damage nucleic acids

Box 1

The formation of cellular ROS

At least four routes can generate hydroxyl radicals ($\bullet\text{OH}$) in a cell^a.

1. Most reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are generated in the mitochondria. ROS genesis *in vivo* in the mitochondria occurs during oxidative metabolism

- a. Reduction of oxygen to water:

- b. The Haber—Weiss reaction:

- c. The Fenton reaction (catalyzed by Fe^{2+} and other transition metals):

2. NADPH oxidase can generate cytosolic ROS in activated inflammatory cells:

3. Nitric oxide reacts with oxygen, superoxide anion ($\bullet\text{O}_2^-$) or thiol compounds, and generates reactive nitrogen species, peroxynitrite or S-nitrosothiol (S-nitroso glutathione)^b, respectively. Peroxynitrite has a half-life of 1–2 s and a long diffusion distance of $\sim 100\ \mu\text{m}$ (Ref. c). Therefore, it readily enters the nuclei or crosses cell membranes and acts in cells other than those in which it is generated.

Arginine $\rightarrow$ [neuronal nitric oxide synthase, Ca^{2+} , NADPH] $\rightarrow$ citrulline + NADP + NO

4. The energy from ionizing radiation causes the formation of cation radical (including H_2O^+ in all molecules, $\bullet\text{OH}$ is formed by the transfer of cation radical to neighboring molecules.

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in the nucleus. For example, mice with either a knockout of neuronal nitric oxide synthase or treated with 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase, are protected against neuronal death in experimental models of brain injury^{17–22}. It has also been reported that 7-nitroindazole reduces damage to nucleic acids in three models of brain injury^{23–26}.

ROS, probably the superoxide anion, hydroxyl radical, singlet oxygen and radical nitric oxide can damage nucleic acids, proteins and lipids, and can impair gene expression in bacteria, yeast and mammalian cells grown in culture^{27–29}. In the brain, elevated ROS can cause oxidative damage to all cellular components by chemically modifying nucleic acid bases and causing single- and double-stranded breaks in the DNA backbone and breaks in the glycosylic bond between ribose and individual bases (abasic or AP site)^{30–33} (Fig. 1). In addition, ROS can cause either DNA strands or protein and DNA to fuse (crosslink). These changes are known as oxidative DNA lesions (ODLs) or oxidative RNA lesions (ORLs) and, if not repaired, they terminate chain elongation or alter the coding properties during transcription (gene expression) or replication. Examples of ORLs/ODLs detected in injured brains are shown in Fig. 2. These lesions mimic those found after ionizing radiation^{34,35} and some have been demonstrated in CNS diseases^{36–39}. In this communication, we will review our current understanding of ORLs/ODLs in the brain and the consequences of such lesions.

Oxidative lesions

Approximately 70 different types of ORL and ODL, including those of free nucleic acids, can be generated by ROS *in vitro*^{40,41} and several assays have been used to detect ORLs/ODLs in the brain (Table 1). Many investigators have detected an increase in single-stranded breaks bearing 3'-OH ends in injured brains. However, the detection of single-stranded breaks with 3'-OH ends might not reliably assay DNA damage, because they could be formed in a number of ways. These include: (1) by a breakage between the ribose and the phosphate group^{42,43} (Figs 1:3a); (2) as an intermediate of DNA repair (Fig. 3); (3) as Okazaki fragments in the replication fork; and (4) as digestion products of nucleases during DNA fragmentation⁴⁴. DNA breaks with 3'-OH ends resulting from repair and replication are part of normal cellular processes and generally are not considered to be DNA damage. Alternatively, the presence of 8-hydroxy-2'-deoxyguanosine in DNA (also known as 8-hydroxyguanosine in RNA) is a reliable marker of base modifications, because it is detected consistently after oxidative stress generated by various conditions^{16,23,26–34}. The detection of base modifications are the preferred indicators of ORLs/ODLs (Refs 47, 48) and can be used to investigate some features associated with CNS disorders^{37,39,47,48}. Because of the large number of lesions, we will limit our discussion to the most reliable types of ORLs/ODLs.

Under normal physiological conditions, an 'average' human cell with a mass of 10^{-9} gram and a volume of $10\ \mu\text{m}^3$ uses $\sim 1 \times 10^{12}$ molecules of oxygen per cell per day and generates 3×10^9 molecules of hydrogen peroxide per cell per hour³⁴. It has been estimated that $\sim 2 \times 10^4$ ODLs occur in each human genome per day^{45,46} and that normal brain contains $\sim 1 \times 10^5$ 8-hydroxy-2'-deoxyguanosine per genome^{31,32}. This is the burden of repair, or a tolerance of gene damage in each normal living cell and represents a balance between the formation and removal (repair) of ODLs.

Several laboratories have reported a >5-fold increase in ODLs of the brain after a short period of cerebral ischemia^{16,31,32}. The activities of DNA repair increase threefold in ischemic brain and they have the capacity to remove ~85% of ODLs within three hours of reperfusion^{1,23,24,31,49,50}. However, the load of ODLs may still exceed the capacity for repair. If one also considers other types of ODLs (Fig. 2), the burden of repair may be more than fivefold. For example, there are at least three lesions in each copy of the *c-fos* gene in animals after cerebral ischemia, compared to one lesion, or fewer, in ten copies of the same gene under normal conditions^{23,49}. The elevated burden is likely to be similar for other nuclear genes³¹, although different genes may be differentially damaged or repaired²⁹.

DNA repair pathways

Studies using cell cultures reveal the complexity of DNA-repair pathways in normal cells. Repair is believed to occur in DNA only because of its double-stranded structure (to serve as a template) and the presence of DNA-dependent DNA polymerases. The major excision—repair pathways are the nucleotide-excision-repair pathway and the base-excision-repair pathway (Box 2). In very general terms the removal of ODLs by the base-excision-repair pathway (Fig. 3) requires recognition and excision of lesions from the DNA double helix by endonucleases or glycosylases^{49,51} (Step 1), followed by hydrolysis mediated by AP endonucleases (Step 2) and clipping of the ribosephosphate moiety to generate a gap for repair synthesis using DNA polymerases (Steps 3 and 4). The repair process is completed by the ligase that connects new and old DNA strands (Step 5). In the nucleotide-excision-repair pathway, DNA lesion-specific endonucleases excise the damaged and adjacent nucleotide, followed by repair synthesis of at least 20 nucleotides in one repair patch. However, base-excision repair generally excises the damaged base [(3a) Fig. 3], and incorporates one nucleotide in the presence of DNA polymerase- β (4a), but no more than 10 nucleotides in the absence of functional DNA polymerase- β (3b and 4b). The repair synthesis is mediated by DNA-dependent DNA polymerase- α , - β , - δ , - ϵ and - γ , with DNA polymerase- β considered to be the most abundant DNA polymerase in the brain. These DNA polymerases act on the 3'-OH end of single-stranded breaks to incorporate the correct dNTP directed by the sequence in the template. In mitochondria, DNA polymerase- γ is responsible for DNA replication and repair synthesis. It is reported that DNA ligation could be dependent on the presence of poly (ADP-ribose) polymerase (PARP) because an inhibition of PARP alters the rate of repair²⁶. Nevertheless, the mechanism of this action is unclear in the brain. Breaks in DNA strands after brain injury activate PARP and cause the depletion of nicotinamide adeninedinucleotide (NAD, a substrate of PARP) and ATP. Base-excision repair is disrupted with PARP-bound DNA (Ref. 52). However, the increase of sister-chromatid exchange in PARP-deficient cells suggests that binding of PARP to DNA is required for orderly ligation of DNA. Activation of PARP enhances necrosis but reduces apoptosis in the brain after injury^{53,56}. Thus, the participation of PARP in gene repair in the brain may be a double-edged sword.

The removal of mismatched bases or large lesions has been described in differentiated neurons in culture⁵⁷ (termed mismatch repair or global repair, respectively, Box 2) although it is not known whether all of these repair pathways are active *in vivo*. Moreover, as some genes are downregulated in cells grown in culture, repair pathways that are absent in culture could be present *in vivo*.

Gene-repair pathways are probably present and functional in the brain, because several proteins that are involved in all types of repair are induced by brain injury and cerebral ischemia^{14,49,58–62}. Evidence of DNA repair has been presented in the brain after stroke, cerebral ischemia or hypoxia^{1,23,31}. Following cerebral ischemia, the ischemia-induced ODLs in the transcribed strand of the *c-fos* gene are repaired initially more slowly than the non-transcribed strand, but almost all are repaired within 2–3 hours^{23,49}. Many investigators have reported a difference

in the repair capacity in aging animals or in individuals with particular neurological disorders^{47,48,59}. While elevated ODLs are reported in mitochondria from the brains of Alzheimer's patients³⁹, others observed elevated ORLs but no detectable ODLs in the cytoplasm³⁷. The rate of ODL repair in nuclear and mitochondrial DNA might be similar in the brain^{1,23,31,49,50}.

When the burden of repair in the brain is increased by fivefold after 30 minutes of cerebral ischemia, the frequency of somatic cell mutations resulting from repair errors is increased from the 'background' frequency of 1.5 mutations per 10⁵ genes to 7.7 mutations per 10⁵ genes in a *lacI* reporter gene in a transgenic Big Blue mouse strain³¹. Therefore, the frequency of mutations in somatic cells *in vivo* correlates positively with ODL levels in the brain. Mutation is randomly distributed in this reporter gene, but approximately 80% occur at G or C bases, with C to T transitions and frameshift mutations. Errors during DNA synthesis *in vitro* in the presence of purified DNA polymerase- β consist mainly of one-base deletions or insertions, which result in frameshifts⁶³. The causal role of DNA polymerase in the formation of frameshifts, however, remains to be established in injured brains.

Frameshift mutations are observed in postmitotic neurons⁶⁴. Mutations have been identified in the gene encoding α -synuclein and the ubiquitin-like *parkin* gene in autosomal recessive juvenile Parkinsonism^{65,66}, in the gene encoding the amyloid precursor protein in conditions related to premature aging⁶⁷, and in the gene encoding superoxide dismutase in familial amyotrophic lateral sclerosis^{68–70}. Some of these include nonsense or deletion mutations. Mutagenesis in somatic cells is rare, but might cause a deleterious effect to the cells if it occurs in an essential gene that has already had a germline mutation in one of its two copies of chromosomes (heterozygote or carrier of a mutation). ODLs accumulate in mitochondria and the frequency of spontaneous mutations in a nuclear gene doubles in the liver of animals lacking the glycosylase that repairs ODLs (oh⁸G, FapyGua, oh⁵C and AP sites, see Fig. 2)^{71,72}. In humans deficient in removing 8-hydroxy-2'-deoxyguanosine neurological disorders develop earlier than in the general population^{39,47}. These studies suggest that a deficiency in DNA repair could upset the balance between ODL formation and repair, and lead to neurological dysfunction.

A major role of DNA repair is to ensure transcription of mRNA from intact genes (Fig. 4). Gene transcription might be pivotal in maintaining neuronal viability after ischemia—reperfusion. Gene activation is induced by oxidative stress^{73–81}, and the genes undergoing transcription contain ODLs (Refs 23–25,31–33,49,50). It is believed that a gene with strand breaks or crosslinks is unable to serve as a template during transcription and that the cell must repair these lesions before transcription can occur²⁹. However, several investigators have reported an elevation of gene transcripts during the first few hours of reperfusion when strand breaks are detected⁴². Although DNA strand breaks and crosslinks inhibit transcription, there is evidence suggesting that modified bases change the coding properties so that most polymerases insert random nucleotides opposite the base, and that adenine nucleotide is most likely to be inserted opposite the 8-hydroxy-2'-deoxy-guanosine lesion during RNA transcription^{82,83}. This 'by-pass insertion' could be another mechanism that generates faulty transcripts with mismatched sequences. What is the probability that a full length mRNA be transcribed from its nuclear gene with ODLs?

One example is the expression of activator protein-1 (AP-1), a transcription factor, which is composed of proteins encoded by *c-fos* and/or other immediate early genes and *trans*-activates late effector genes that are either detrimental^{75,84} or salutary to cell viability^{80,81,85,86}. Under normal physiological conditions, little mRNA encoding FOS is detected in the brain. Levels increase following cerebral ischemia, probably as a result of transcription of its nuclear gene. One hypothesis is, if the nuclear genes contain ODLs, the newly transcribed mRNA could be

faulty. This proposal is supported by the finding that it takes over one hour for the increase in FOS/AP-1 activity to become apparent following injury-induced increase in *c-fos* mRNA (Refs 87,88). It is thought that this delay is caused by an inhibition as a result of translation blockage or a reduced global protein synthesis¹⁵. This blockage coincides with the duration of detecting excessive ODLs in *c-fos*, that is, the blockage expires when the majority of ODLs in nuclear genes are repaired^{23–25,49,50}.

Box 2

DNA-repair pathways in mammals

Nucleotide-excision-repair (NER) pathway^{a,b}

The NER excises several nucleotides (20–30 nucleotides) surrounding the lesion in each repair patch. There are two modes: (1) transcript-coupled repair (TCR) mode, which has a preference for repair of the transcribed strand, most likely the exons of active genes. Enzymes of TCR can also be gene activators; or (2) global-excision repair mode, induced by p53, repairs bulky lesions without preference for either strand. Unless there is degeneration of the blood—brain barrier, as in some diseases, chemicals that induce the bulky lesions (e.g. fusion of adjacent bases [dimers] and strands [crosslinks], strand breaks) do not enter the brain.

Base-excision-repair (BER) pathway^{c–e}

Conventional BER excises and repairs one base (Fig. 3, steps 3a and 4a). DNA polymerase- β is thought to perform repair synthesis during BER. Because DNA polymerase- β is the most abundant DNA polymerase in the brain, BER could be the major repair pathway in this tissue. In the absence of functional DNA polymerase- β an alternative BER pathway that requires several enzymes may operate (Fig. 3, steps 3b and 4b). Thus far, thymine glycol is the only ODL that is preferentially repaired on the transcribed strand.

Mismatch repair^{f,g}

This repair corrects a structure in duplex DNA composed of two unmodified nucleotides in a non-Watson—Crick base pair of mismatched but undamaged bases that are either incorporated during DNA replication and repair synthesis, or that result from deamination of cytosine.

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Conclusion

We have discussed some aspects of the emerging field of gene damage and repair in the genetic material of the brain after cerebral ischemia—reperfusion. ORLs/ODLs cause adverse effects on the processes of replication, transcription and translation, therefore they affect every level of cell function. Excessive ORLs/ODLs might cause deleterious effects ranging from mutation to cell death.

Although the effect of ORLs might be transient as a result of the short half-life of RNA, they might have profound effects on the recovery of cells after brain injury. A reduction of ORLs, using 7-nitroindazole, increases neuronal viability suggesting that non-functional mRNA might affect the endogenous response to recovery from the acute conditions of brain injury and stroke²⁵. Reducing non-functional mRNA might increase translation efficacies for functional proteins. Moreover, because RNA is not repaired, the ORL could be stable for the life of RNA. Future direction includes the detection of ORLs as a marker of oxidative damage in cells from various neurological disorders³⁷.

Nuclear genes can be crucial targets for ODLs because they are present in 1–2 copies in each mammalian cell. If the ODL is not repaired, it will alter the transcripts for the remainder of the life of the cell. The repair of ODLs in at least three nuclear genes does not appear to follow the transcription-coupled mechanism^{23,31}. Recent evidence suggests that recovery appears to correlate with the ability of the affected tissue to remove or repair oxidative damage^{17–25,89}. It is clear that during initial reperfusion phase there is a period in which damage exceeds repair. What is the effect on the transcript generated from the gene that is activated but has not yet been completely repaired during oxidative stress? Deciphering the importance of nucleic acid damage and mechanisms of repair could highlight targets for therapeutic limitation of the spread of ischemic damage.

We also discussed that errors in repair appear to occur preferentially at GC sequences and mutations are mostly frameshift mutants in a transgenic animal strain. There is a need to investigate the relationship between frameshift mutations after brain injury and pre-existing heterozygotic germline mutation, especially in those genes that are associated with endogenous responses for neuroprotection, such as ROS scavengers and gene activators, and those genes that are implicated in causing neurological disorders. The identification of damage to genetic material early in the ischemic process might allow development of appropriate therapeutic interventions.

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Fig. 1.

Schematic illustration of the formation of DNA and RNA lesions. Hydroxyl radicals can attack nearby DNA at several places, as indicated by the arrows. Some lesions resulting from the attack are listed in Fig. 2. Numbers represent the position of attack by reactive oxygen species. (1) attack on the base which produces base modifications. (2) attack on the glycosidic bond which produces sites without base (AP sites). (3) attack on the phosphate bond (P), which produces DNA strand breaks, either (3a) with 3' hydroxyl ends (3'-OH) or (3b) with 3' phosphate ends (3'-PO₄). (4) attack on the ribose which produces 3' phosphoglycolate ends (3'-PG).

Fig. 2.

Nucleic acid modifications identified in the brain after oxidative stress. (a) 8-hydroxyguanine [Oh⁸G], the hydrogen on the C-8 carbon of the guanine base (also termed 8-oxo-7,8-dihydroguanine) is replaced with a hydroxyl group. (b) 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (FapyGua), an open ring results from a broken bond between the C-8 and C-9 carbons in the guanine base. (c) 8-hydroxyadenosine (Oh⁸A), the hydrogen on the C-8 carbon of the adenine base is replaced with a hydroxyl group. (d) 5-hydroxycytosine (Oh⁵C), the hydrogen on the C-5 carbon of the cytosine base is replaced with a hydroxyl group. (e) AP site in DNA. (f) and (g) DNA breaks with two different termini. Most of these lesions have been reported in brain injury models, including the forebrain ischemia—reperfusion (experimental cardiac arrest) model, the focal cerebral ischemia model (experimental stroke), the seizure model and the traumatic brain injury model^{1,14,16,23,31-33}.

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Fig. 3.

The two distinct base-excision-repair pathways in mammalian cells. To repair a modified base, the glycosylic bond linking the deoxyribose (dR) and the damaged base (G^*) is excised via glycosylase⁶² (1) to generate an abasic (AP)-site intermediate. The damaged base can also be released spontaneously, without enzyme. The DNA backbone 5' to the AP site is cleaved by AP endonuclease in Step 2, to generate a single-stranded break with 3' hydroxyl and 5'-deoxyribose phosphate (dRP) termini that can be repaired by one of two pathways. In the classical pathway, dRP is removed by DNA polymerase-β (DNA pol-β; 3a) followed filling of the one-nucleotide gap (4a) and DNA ligation (5). The alternative pathway (3b and 4b), which occurs in the absence of functional DNA pol-β, involves repair synthesis by proliferating cell nuclear antigen (PCNA), DNA pol-δ or -ε and flip endonuclease (FEN). AP endonuclease, DNA pol-β and DNA ligase I form a multiprotein complex⁵¹ in which DNA ligase I can be replaced by DNA ligase III plus complementary factor XRCC1.

Fig. 4.

Proposed mechanism by which ODLs in *c-fos* could delay trans-activation of late genes by producing faulty gene expression without repair (thin red arrows). Repair processes (green arrows) will enhance the expression of intact mRNA (Ref. 25). Abbreviations: ROS, reactive oxygen species; NGF, nerve growth factor.

Table 1Assays that investigate DNA damage and repair in the brain or CNS derived cells^a

Name of assay	Purpose	Refs
<i>In vivo</i> mutation assay	The Big Blue transgenic mice with a reporter gene that can be easily isolated for detection of gene mutations.	31
Single-strand conformation polymorphism (SSCP)	Mutation screening that detects altered transcript from specific genes.	31
HPLC or GCMS	Detection of modified nucleosides or bases in purified DNA.	16:31:32, 39:40
Immunohistochemistry	IgG or IgM against 8-hydroxy-2'-deoxyguanosine/8-hydroxyguanosine.	23:26:33, 37:49
Gene-specific repair using fragment shift	The presence of ODLs is detected by the ability of the repair enzyme to remove ODLs in purified DNA. An absence of the gene during hybridization in alkaline blot using gene specific probe signifies the presence of ODLs.	21:31:56
DNA lesions detected as sensitive sites to DNA repair glycolylases or nucleases <i>in situ</i>	Detection of specific DNA lesions in brain tissue as sensitive sites to the Fpg protein or exonuclease III (FPGSS or EXOSS, respectively) enzymes followed by post-labeling using DNA polymerase.	24:50
Repair activity assay	Expression of enzymes that repair base modifications, e.g., 8-hydroxy deoxyguanosine or AP sites.	2:49
DNA strand-break assay	DNA polymerase-I for detecting SSBs with 3'OH ends.	42
<i>In vitro</i> PCR	Detection of DNA lesions that do not support PCR in purified DNA.	1
The comet assay	The presence of a comet tail represents the presence of SSBs.	35:43

^a Abbreviations: GCMS, gas chromatography and mass spectrometry; HPLC, high-pressure liquid chromatograph; ODL, oxidative DNA lesions; SSB, single-stranded break.