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Mitochondrial base excision repair pathway failed to respond to status epilepticus induced by pilocarpine

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ABSTRACT

Oxidative damage to mitochondrial DNA (mtDNA) has been implicated as an important mechanism underlying mitochondrial deficiency in epileptic seizures. In focusing on the role of the DNA repair pathway, we determined the response of the mitochondrial base excision repair (mtBER) pathway in pilocarpine-induced status epilepticus (SE) in hippocampi of male Wistar rats. The expression of 8-oxoguanine DNA glycosylase (OGG1) and polymerase γ (polγ) was decreased at both the cellular mRNA and mitochondrial protein levels at 3, 9 and 25 h after the onset of SE. The mRNA and protein levels of APE1 were maintained, but the mitochondrial protein level decreased at 3 and 9 h and recovered at 25 h. Therefore, the mtBER pathway failed to respond to SE induced by pilocarpine. The failure of mitochondrial import might be an important factor responsible for the lowered mtBER enzymes in mitochondria. We hypothesize that the down-regulation of mtBER enzymes may aggravate mtDNA damage and mitochondrial deficiency after the onset of SE.

Accumulating evidence indicates that reactive oxygen species (ROS) may be a contributing factor in the generation of epileptic seizures [26,30], and oxidative damage to susceptible targets (protein, lipids and DNA) has been assessed by several studies [10,12,18]. In contrast to nuclear DNA (nDNA), mitochondrial DNA (mtDNA) is lack of the protection of histones, and the close proximity to electron transport chain, which is the major source of $O_2^{\bullet -}$ production, makes it more vulnerable [36]. Mammalian mtDNA codifies a small, but essential number of polypeptides of the oxidative phosphorylation (OXPHOS) system. mtDNA damage might influence the expression of these peptides and subsequently contribute to mitochondrial OXPHOS deficiency [35,40]. Oxidative damage to mtDNA has been implicated in mitochondrial deficiency in epileptic seizures [15,18,22]. Therefore, DNA repair in the mitochondria should be extremely important.

Some studies have demonstrated a potential role for several DNA repair pathways in neuroprotection against seizures [3,7,11,18,29]. The base excision repair (BER) pathway is a major DNA repair pathway responsible for recognizing, excising, and repairing oxidative DNA damage [42]. It is important to stress that BER pathway exists in the mitochondria as well as nucleus [4,23]. As a consequence of oxidation, mtDNA can also exhibit strand breaks, DNA–DNA and DNA–protein cross-linking and base modification like nDNA [6,8]. However, compared with various nuclear DNA repair pathways, only the BER pathway has been shown to function in the mitochondria [24] and thus is critical in the defense of mitochondrial oxidative stress. However, the mitochondrial BER (mtBER) pathway has been rarely explored in epileptic seizures.

The mtBER pathway involves a highly coordinated process catalyzed by the sequential actions of DNA repair enzymes [4,23,42]. The first step of the pathway is carried out by a DNA glycosylase (for example 8-oxoguanine DNA glycosylase, OGG1), which recognizes and removes the damaged base leaving an abasic site (AP site), then the AP site is cleaved by apurinic/apyrimidinic endonuclease1 (APE1), and the base gap is filled in by DNA polymerase γ (polγ) and ligated by a DNA ligase. Nuclear and mitochondria-specific DNA glycosylases and APE1 are encoded by the same nuclear genes and transported respectively. To exclude the influence of the nuclear BER pathway, we isolated mitochondrial fractions and detected the expression of BER enzymes in mitochondria.

Since mtDNA damage caused by ROS may participate in epileptogenesis [15,18,22], we focused on the role of mtDNA repair pathway in epilepsy. We aimed to determine the response of

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Table 1
Primer sequences.

Gene	Primer sequences (5′ ~ 3′)	Annealing temperature (°C)	Length (bp)
OGG1	GATGTCACTTATCATGGCTTCC CAACTTCCTGAGGTGGGTC	56	75
APE1	GGTGCTCCAGACGCCTAA AGCCCATCCACATTCCAG	56	248
polγ	ATGTCCTTGTGGGTGCTG GCGGCTGTCTTACTGTGC	58	162
β actin	GACAGGATGCAGAAGGAGATTACT TGATCCACATCTGCTGGAAGGT	58	142

the mtBER pathway in pilocarpine-induced status epilepticus (SE), which is characterized by elevated oxidative stress [10,12].

The study was performed in accordance with international guidelines and approved by the Chinese Institutional Animal Care Committee. Animals were kept in standard conditions (12 h light and dark cycle) with free access to food and water during all experimental time period. The number of animals used was minimized for the statistical analysis. Scopolamine methylnitrate (1 mg/kg) was injected subcutaneously 30 min before pilocarpine administration to prevent peripheral cholinergic effects. Seizures were induced by pilocarpine injections as reported previously [43]. Adult male Wistar rats (Experimental Animal Center of Shandong University) weighing 200–250 g were given lithium chloride intraperitoneally (3 mEq/kg, i.p.). Twenty hours later, experimental rats received pilocarpine (30 mg/kg, i.p. Sigma, St. Louis, MO, USA) and control animals received normal saline at the same volume. The onset of SE was determined by the presence of continuous stage 4–5 level seizures according to Racine [32]. Seizures were allowed to last for 60 min and then diazepam (10 mg/kg, i.p.) was injected to shorten the duration of SE and reduce the mortality rate. Control rats were treated with the same protocol, but with saline instead of pilocarpine. Rats were decapitated at 3, 9, and 25 h after the onset of SE.

RNA isolation from the snap frozen hippocampi ($n = 4$ per group) was done using TRIzol Reagent (Invitrogen, carlsbad, CA, USA). 5 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Fermentas, Glen Burnie, MD, USA). LightCycler 2.0 (Roche, Mannheim, Germany) was used for detecting real-time PCR products specific for *OGG1*, *APE1*, *polγ* and *βactin*. Primer information is in Table 1.

Rats were sacrificed by decapitation and hippocampi were removed and quickly placed in chilled isolation medium (0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 250 μg BSA/ml). The tissues were thoroughly washed and 10% (w/v) homogenates were prepared. The nuclei and cell debris were precipitated by centrifugation at 800 × *g* for 10 min and discarded. The supernatant was subjected to a further centrifugation at 10,000 × *g* for 15 min. The pellets were suspended in 3% Ficoll 400 and gently layered onto 6% Ficoll 400. The gradients were centrifuged at 10,400 × *g* for 25 min. The pellets were washed in isolation medium and stored at −80 °C. All steps were carried out at 0–4 °C.

Protein was extracted from the whole hippocampi ($n = 4$ per group) and the mitochondrial fractions ($n = 4$ per group), and was measured by BCA protein assay kits (Beyotime, Jiangsu, China). 30 μg of total protein was loaded into each lane of an SDS–polyacrylamide gel and separated by electrophoresis. Prestained standards (Fermentas, Hanover, US) were used as molecular weight markers. Proteins were transferred to polyvinylfluoride membranes (Bio-Rad, Hercules, CA, USA) and then incubated with primary antibodies against cytochrome oxidase subunit IV (COX IV) (1:500; Santa Cruz, CA, USA), OGG1 (1:500, Abcam, UK), APE1 (1:4000, Abcam, UK), polγ (1:100, Santa Cruz, CA, USA), β actin (1:5000, Santa Cruz, CA, USA) at 4 °C overnight, then horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology). The protein band was visualized by enhanced chemiluminescence kits (Amersham, Buckinghamshire, UK). The reactions were analyzed by FluorChem HD IS-9900 Imaging System, and band intensities were quantified with an image analyzer (Alpha Innotech, San Leandro, CA, USA).

Data are expressed as mean ± standard deviation. One-way ANOVA and the Newman–Keuls test were used for statistical analysis of the results as appropriate. Significance level was set at $p < 0.05$.

About 2–8 min after pilocarpine administration, video monitoring of experimental animals revealed oro-facial movements, salivation, eye-blinking, twitching of vibrissae, and then symptoms such as stereotypical oral, masticatory movements, chewing, and tremors. The seizures evolved to SE in 83.1% of rats (64/77). The time from pilocarpine injection to the first onset of stage 4 SE was 38.5 ± 18.7min. Mortality due to SE at 3, 9, and 25 h was 5.3% (1/19), 9.1% (2/22) and 21.7% (5/23) respectively. Stages 4–5 seizures lasted for 0.73 ± 0.341 h in 3 h after the onset of SE.

Real-time PCR analysis demonstrated lower expression of both *OGG1* and *polγ* in experimental hippocampi than control hippocampi at 3, 9, and 25 h after the onset of SE, with no significant difference between experimental and control rats in *APE1* level (Fig. 1A). The protein level of APE1 in the hippocampi was constant in each group (Fig. 1B).

To determine the response of the BER pathway in mitochondria, we isolated mitochondrial fractions. The protein level of OGG1 was significantly reduced at 3, 9, and 25 h after the onset of SE, and so was the immunoreactivity of polγ compared with control (Fig. 2). However, APE1 decreased at 3 and 9 h and then returned to the control level at 25 h (Fig. 1A and B).

In this study we found that polγ and OGG1 were decreased in expression at both the cellular mRNA and mitochondrial protein levels in rat hippocampi at 3, 9, 25 h after the onset of SE. Despite a steady expression of APE1 at both the mRNA and protein levels in hippocampi, mitochondrial APE1 protein expression decreased at 3 and 9 h and recovered at 25 h. Therefore, mtBER enzymes failed to respond to SE induced by pilocarpine in rat hippocampi.

Oxidative stress has previously been shown to induce DNA repair proteins [17,24], but the underlying mechanism is complicated and not thoroughly understood. A key question is how oxidative stress, which is implicated in various diseases, can differently regulate BER proteins. For example, APE1 level is decreased after transient global ischemia and transient spinal cord ischemia [14,20,34], whereas ischemic preconditioning in the rat brain enhances the BER repair pathway and OGG1 is upregulated after forebrain ischemia reperfusion [25,27]. Similar discrepant findings were observed in seizure models. In this study the decrease in mtBER enzymes in rat hippocampi might be due in part to a transcriptional regulation after the onset of SE, as demonstrated by the decreased mRNA levels of polγ and OGG1. So the BER pathway failed to respond to the elevated oxidative stress, which is widely accepted as one of the factors responsible for the neuronal damage in pilocarpine-induced SE. In contrast BER enzymes are elevated in kainic acid (KA)-induced acute seizures [18,31]. It seems that the elevated oxidative stress is not the only regulatory factor in seizures. Different models show different processes underlying epilepstogenesis: level of oxidative stress, severity of neuronal damage, and the involved signaling pathways. As well, different models mimic different conditions. For example, the KA and pilocarpine models are considered similar classic models of temporal lobe epilepsy (TLE), but recently the pilocarpine or LiCl-pilocarpine model of SE was revisited for study of SE associated with systemic inflammatory processes, by the observation of elevated ILβ and alteration in blood–brain barrier permeability [28,39]. The gap between oxidative stress and modulation of the BER pathway needs further exploration.

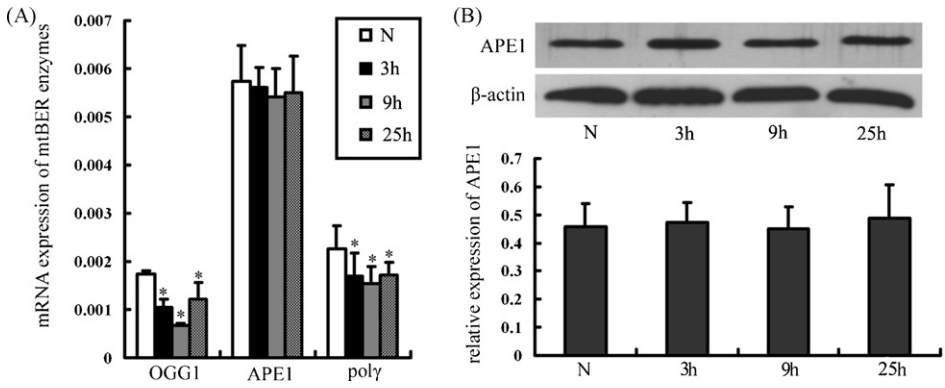

Fig. 1. (A) Real-time PCR analysis of mRNA expression of *OGG1*, *APE1* and *polγ*. (B) Western blot analysis of APE1 protein expression in whole hippocampi. β actin served as a loading control. Data are expressed as mean ± SD of at least 4 independent animals. $^{*}P < 0.05$, compared with the control group.

Mitochondria contain ~1000 different proteins, and most of these proteins must be imported from the cytosol. Nuclear and mitochondria-specific DNA glycosylases and APE1 are encoded by the same nuclear genes. The mitochondrial enzymes lack the nuclear localization signal (NLS) and contain an N-terminal mitochondrial targeting sequence (MTS). These preproteins are recognized and sorted by protein translocases present in both the outer and inner mitochondrial membranes [1]. Several stimuli, including contractile activity of skeletal muscle, thyroid hormone treatment, muscle cell differentiation, and mtDNA defects, can alter the expression of the import pathway components, ultimately leading to a change in protein import rate and mitochondrial phenotype [5,16,19,37]. The distribution of APE1 was also revealed in several cell types, and recent researches indicate that APE1 could differentially translocate among nucleus, mitochondria and cytoplasm in response to oxidative stress [2,13,38], but little is known about the underlying regulatory mechanism. We found APE1 reduced in mitochondria in experimental groups, with no difference from controls in the whole hippocampus. It is plausible that the sophisticated translocation and assembly devices might be influenced to some extent after the onset of SE and the import of mtBER enzymes might be involved.

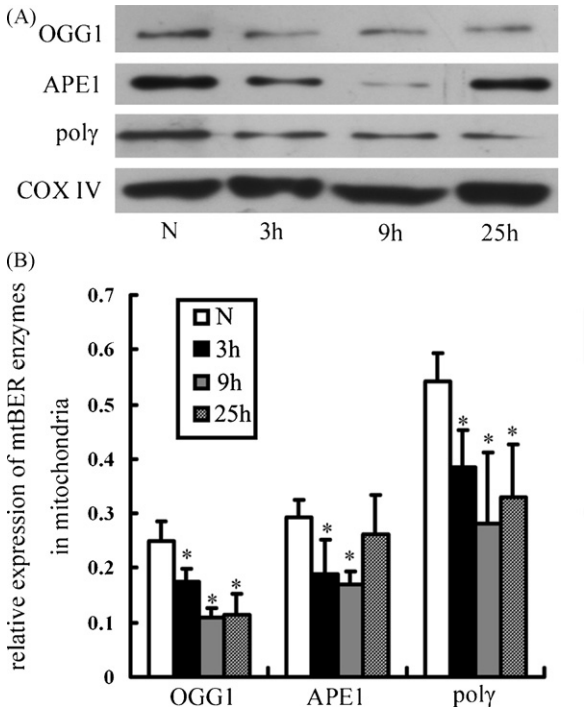

Fig. 2. (A) Western blot analysis of protein expression of OOG1, APE1 and polγ in mitochondrial at the indicated times; COX IV served as a loading control. (B) Quantitative representation of protein expression. Data are expressed as mean ± SD of at least 4 independent animals. $^{*}P < 0.05$, compared with the control group.

The role of the BER pathway in the development and maintenance of the central nervous system is supported by the down-regulation of enzymes involved in the BER pathway contributing to brain injury [9,20,41] and upregulation promoting neuronal survival [25,33]. The mtBER pathway represents an endogenous protective mechanism against mitochondrial oxidative damage in pathophysiological events. Neuronal survival depends upon a fine balance between oxidative damage and the damage repair system. Increased lipid peroxidation, decreased glutathione peroxidase content, and excessive free radical formation occur during SE induced by pilocarpine, indicating a high level of oxidative stress after the onset of seizures [10,12]. Recent studies confirm that mitochondrial, not nuclear, DNA is the source of seizure-induced increased 8-OHdG level, an index of oxidative DNA damage [21]. Together with upregulated ROS levels, the failure of the mtBER response could lead to further mtDNA damage and mitochondrial deficiency after the onset of SE. Seizure-induced accumulation of oxidative mtDNA lesions and resulting somatic mtDNA mutations could over time render the brain more susceptible to subsequent epileptic seizures. Confirmation of this presumption needs further research and might provide new insight into epileptogenesis.

In conclusion, our results show that mtBER enzymes failed to respond to SE induced by pilocarpine in rat hippocampi. The failure of mitochondrial import might be an important factor responsible for the lowered mtBER enzymes in hippocampal mitochondria. Down-regulation of mtBER enzymes may aggravate mtDNA damage and mitochondrial deficiency after the onset of SE.

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