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Tert-butylhydroquinone compromises survival in murine experimental stroke

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ABSTRACT

Tert-butylhydroquinone (tBHQ), an Nrf2 signaling pathway inducer that is widely used as a food additive in the U.S., prevents oxidative stress-induced cytotoxicity in neurons. This study assesses the effects of tBHQ on ischemic stroke outcomes in mice. We measured infarct size, neurological deficits, and brain volume after tBHQ treatments in murine permanent middle cerebral artery occlusion (pMCAO) model in vivo. Further, we evaluated the regulation of tBHQ on mitochondrial function in cerebrovascular endothelial cells in vitro, which is critical to the blood–brain barrier (BBB) permeability. Our results demonstrated that tBHQ increased post-stroke mortality and worsened stroke outcomes. Mitochondrial function was suppressed by tBHQ treatment of cerebrovascular endothelial cells, and this suppression was potentiated by co-treatment with lipopolysaccharide (LPS), the bacterial mimic. These data indicate that tBHQ-exacerbated stroke damage might due to the compromised BBB permeability in permanent stroke.

1. Introduction

As reported by the World Health Organization, over 15 million people a year suffer from stroke worldwide (Weir & Dennis, 1997). In the United States, stroke is the fifth leading cause of mortality and the leading cause of long-term disability (Roger et al., 2012). Ischemic stroke accounts for approximately 80–85% of all cases, which is caused by the disruption of cerebral blood flow and lack of oxygen and glucose to the affected area due to a clot. Currently, the only Food and Drug Administration approved treatment for ischemic stroke is tissue plasminogen activator (tPA), which is a thrombolytic therapy to break up the clot. Properly titrated use of tPA improves clinical outcomes. However, less than 5% of patients receive tPA (Miller et al., 2011). Overall, the current therapeutic strategy for ischemic stroke is not optimal.

Brain injury following cerebral ischemia develops from a complex series of pathophysiological events that evolve in time and space (White et al., 2000). Energy failure leads to intracellular ionic imbalance, excessive production of oxygen radicals and impairment of mitochondrial function in neurons (Dirnagl et al., 1999; Lee et al., 1999). A considerable body of evidences suggests that excessive production of reactive oxygen species (ROS) is a fundamental contributor to brain damage in ischemic stroke (Manzanero et al., 2013). Even though ROS are short-living compounds, they can initiate complex chain reactions. ROS directly react with a multitude of biological target molecules, such as proteins, lipids and DNA, to produce a wide range of intracellular damage (Imlay, 2003). All of oxidative modified intracellular molecules exert a fundamental impairment of biological function. Not only might ROS induce accidental damage to molecules, but also actively modulate critical subcellular organelle functions, such as endoplasmic reticulum stress and mitochondrial dysfunction (Jacobson, 1996). Many therapeutic strategies have targeted antioxidants to promote neuroprotection during ischemia (Gilgun-Sherki et al., 2002). However, to date, no antioxidative approaches have been approved in the United States. We should note that most of drug candidates tested in the clinical trials of stroke are free radical scavengers that directly react with ROS. This strategy can rapidly eliminate ROS, but it fails to improve endogenous antioxidative status. Overall, the current antioxidative strategy for ischemic stroke therapy is not optimal.

The nuclear factor E2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway controls a battery of antioxidative gene expression. Activation of Nrf2-ARE signaling pathway improves intracellular antioxidant capacity and is critical for the detoxification and elimination of oxygen-derived free radicals. Under normal condition, Nrf2 binding with Kelch-like ECH-associated protein 1 (Keap1), a sensor of intracellular redox status, is inactivated (Itoh et al., 1999). Upon the stimulation of oxidative stress, Nrf2 dissociates with Keap1, translocates into the nucleus and further activates the transcription of ARE-driven genes (Apopa et al., 2008). ARE-driven genes are involved in a battery of antioxidant and phase II enzymes production (Jaiswal, 2004). One

Abbreviations: ARE, antioxidant response element; BBB, blood–brain barrier; FCCP, carbonilcyanide *p*-triflouromethoxyphenylhydrazone; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor E2-related factor 2; OCR, oxygen consumption rate; pMCAO, permanent middle cerebral artery occlusion; ROS, reactive oxygen species; tBHQ, tert-butylhydroquinone; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

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well-characterized Nrf2 inducer is tert-butylhydroquinone (tBHQ). In addition, tBHQ is an antioxidant, used as a food additive for unsaturated vegetable oils as well as many other edible animal fats. The maximal content of a food containing the additive is 0.02% of the oil or fat content (National Toxicology Program, 1997). As an antioxidant, tBHQ can boost intracellular antioxidative capacity immediately after administration, which directly reacts with ROS and prevents oxidative damage. Activation of Nrf2-ARE signaling pathway results in an increase in a wide range of intracellular antioxidant and antioxidative enzyme levels. This can fundamentally reestablish intracellular redox hemostasis. The dual antioxidative activities make tBHQ as a valuable candidate for the treatment of oxidative stress-induced neurodegeneration. Our previous study has shown that tBHQ exerts protection against oxidative stress-induced cytotoxicity in neurons though preserving mitochondrial function (Sun et al., 2015). A published study reported that pretreatment with tBHQ reduced cortical damage and improved functional recovery up to 1 month after ischemia–reperfusion in rats (Shih et al., 2005).

We investigated the effect of tBHQ on a murine permanent middle cerebral artery occlusion (pMCAO) model, a severe ischemic damage. Surprisingly, tBHQ induced a significant increase of mortality and failed to protect stroke outcomes compared to control. Further, we demonstrated that tBHQ significantly increased brain volume in murine pMCAO model. Finally, we found that tBHQ inhibited mitochondrial function of cerebrovascular endothelial cells, which is critical to the blood–brain barrier (BBB) permeability (Doll et al., 2015). BBB disruption can lead to edema and further aggravate the ischemic damage (Kamada et al., 2007). These data suggest that tBHQ exposure leads to mitochondrial suppression-mediated BBB disruption, which exacerbates acute stroke outcomes.

2. Materials and methods

2.1. Animals and experimental treatment

All procedures were conducted according to the criteria approved by the Institutional Animal Care and Use Committees at the West Virginia University (WVU). C57/BL6J male mice (3–4 months old, 25–30 g; Jackson Laboratories) were used for all studies. tBHQ (Sigma, Saint Louis, MO) solution was prepared at 0.116, 0.668 and 6.68 mg/ml in vehicle (1% DMSO in saline). Intraperitoneal injection of tBHQ (0.582, 3.34 or 33.4 mg/kg) was performed every 12 h, starting at 24 h before pMCAO. An equal volume of saline was administered to control mice. To assign pretreatments of mice, we numbered the animals and applied a simple randomization by using excel-generated random numbers. The experimenters were blinded to the pretreatments and data analysis.

2.1.1. Permanent middle carotid artery occlusion

All surgical anesthesia was induced with 4–5% isoflurane until the animal showed no response to a toe pinch and was maintained with 1–2% isoflurane via face-mask in O_2-enriched air. We performed focal cerebral ischemia for 24 h by occlusion of the right middle cerebral artery with a 6.0 monofilament suture (Doccol, Sharon, MA). We used laser Doppler flowmetry (Moor Instruments, United Kingdom) to detect regional cerebral blood flow and confirmed a successful occlusion (>70% decrease in flow). Rectal temperature was maintained at 37 ± 0.5 °C with a warm blanket (Stoelting Co, IL USA) during surgery.

2.1.2. Neurological deficits

Neurological deficit was determined daily before and after tMCAO according to a 0- to 5-point scale neurological score system as published (Doll et al., 2015). 0 = no neurological dysfunction; 1 = failure to extend left forelimb fully when lifted by tail; 2 = circling to the contralateral side; 3 = falling to the left; 4 = no spontaneous walk or in a comatose state; 5 = death. The experimenters were blinded to group allocations.

2.2. Exclusion criteria for the successful animal experiments

The following criteria for successful pMCAO were observed for all animals. (1) Regional cerebral blood flow decreased <70% during occlusion as detected by laser Doppler flowmetry. (2) Surgery time was never over 30 min. (3) Neurological deficits were observed 3 h after MCAO (neurological score 0). (4) Infarction in the MCA territory (striatum) on 2,3,5-triphenyltetrazolium chloride (TTC) staining was seen. (5) There was no subarachnoid hemorrhage on postmortem examination. (6) There was no substantial ambient temperature change (22–24 °C) in the animal facility. Animals that died before the planned time of assessments were postmortem examined for subarachnoid hemorrhage, and the mortality was recorded.

2.3. Analysis of infarct size and brain volume

Mice were euthanized with isofluorane. We removed the brains and cut 2-mm coronal sections with a mouse brain matrix. We stained the sections with 2% TTC (Sigma, Saint Louis, MO) in phosphate buffer solution at 37 °C for 30 min then fixed the tissue in 10% formalin phosphate buffer for digital photograph. We analyzed the digitized images of each brain section using a computerized image analysis software (ImageJ, National Institutes of Health) in a blinded manner. The infarction volume was expressed as a percentage of contralateral cortex, striatum, and total hemisphere. Brain volume for ipsilateral hemisphere (right) and contralateral hemisphere (left) was calculated.

2.4. Cell culture

The bEnd.3 cell line (CRL-2299 from ATCC, Manassas, VA) was originally derived from mouse brain cortex endothelial cells and confirmed by the observed major phenotypic features of the BBB (Doll et al., 2015). Passages 25 to 30 were used in the study. The bEnd.3 cells were routinely grown in high glucose Dulbecco modified Eagle medium (ATCC) supplemented with 10% FCS and 1% penicillin/streptomycin (Hyclone, South Logan, UT) at 37 °C in 5% CO_2 humid atmosphere.

2.5. Oxygen consumption

Oxygen consumption rate was measured at 37 °C using an XF96e extracellular analyzer (Seahorse Bioscience, North Billerica, MA) according to the manufacturer's instructions. Briefly, the bEnd.3 cells were seeded into Seahorse Bioscience XF96e cell culture plates (16,000 cells/well) in 80-μL medium and allowed to adhere and grow overnight. After respective exposures, the media was exchanged 1 h prior to the assay with XF assay medium (Seahorse Bioscience, North Billerica, MA). Oligomycin (1 μM), carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP, 0.5 μM) and antimycin (1 μM) and rotenone (1 μM) (Sigma) were diluted into XF^{e}96 media and loaded into the accompanying cartridge. Injections of the components into the wells occurred at the time points specified. Oxygen consumption rate (OCR) was monitored using a Seahorse Bioscience XF^{e}96 Extracellular Flux Analyzer. To calculate basal respiration, the measurement prior to oligomycin addition was subtracted from OCR measurement after rotenone and antimycin injection. ATP production was measured using the third OCR measurement prior to addition of oligomycin subtracted from OCR after oligomycin injection. Maximal respiration was calculated using the maximal OCR after FCCP injection subtracted from OCR after rotenone and antimycin injection. To calculate spare capacity, the maximal OCR after FCCP injection was subtracted from the measurement prior to addition of oligomycin.

2.5.1. Statistical analysis

The data were shown as means ± SD. Statistical analyses were performed using one-way ANOVA with Tukey's post hoc tests for multiple