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Mannitol-facilitated perfusion staining with 2,3,5-triphenyltetrazolium chloride (TTC) for detection of experimental cerebral infarction and biochemical analysis

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ABSTRACT

A simple method to quantify cerebral infarction has great value for mechanistic and therapeutic studies in experimental stroke research. Immersion staining of unfixed brain slices with 2,3,5-triphenyltetrazolium chloride (TTC) is a popular method to determine cerebral infarction in preclinical studies. However, it is often difficult to apply immersion TTC-labeling to severely injured or soft newborn brains in rodents. Here we report an *in vivo* TTC perfusion-labeling method based on osmotic opening of blood–brain-barrier with mannitol-pretreatment. This new method delineates cortical infarction correlated with the boundary of morphological cell injury, differentiates the induction or subcellular redistribution of apoptosis-related factors between viable and damaged areas, and easily determines the size of cerebral infarction in both adult and newborn mice. Using this method, we confirmed that administration of lipopolysaccharide 72 h before hypoxia–ischemia increases the damage in neonatal mouse brains, in contrast to its effect of protective preconditioning in adults. These results demonstrate a fast and inexpensive method that simplifies the task of quantifying cerebral infarction in small or severely injured brains and assists biochemical analysis of experimental cerebral ischemia.

1. Introduction

Immersion staining of fresh brain slices with 2,3,5-triphenyltetrazolium chloride (TTC) is a simple and popular method for detecting infarction in experimental stroke models (Bederson et al., 1986). TTC, a colorless water-soluble dye, is reduced to a deep red, water-insoluble compound (formazan) predominantly in the mitochondria of living cells, hence distinguishing between viable and infarcted brain tissue after stroke. However, immersion TTC-staining has its limitations because it is often difficult to section unfixed, edematous brains after severe ischemic injury, especially in newborn rodents. While perfusion staining of rodent brains by intracardiac injection of TTC solution was reported, we have found this method to be very inefficient (see Section 3), and the previous TTC perfusion-labeling method was rarely adopted in the literature (Isayama et al., 1991). The lack of a reliable *in vivo* TTC-labeling method has increased the workload of quantifying tissue damage in experimental stroke, and uncertainty in distinguishing the core-versus-peri infarct areas for biochemical analysis.

Interestingly, although intracardiac injection of TTC hardly labels the brain, it stains the heart efficiently and has been used widely to detect experimental myocardial infarction. This disparity suggests to us that the entry of TTC dye to central nervous system may be hindered by the blood–brain-barrier (BBB). To test this hypothesis, we performed TTC perfusion-labeling after osmotic BBB disruption with mannitol (Rapoport and Thompson, 1973; Rapoport, 2000), and found a greatly enhanced brain-staining capacity. Here we report optimization of the TTC perfusion-labeling method that demarcates infarction in both adult and newborn mouse brains. Further, we show that the *in vivo* TTC-labeling method is compatible with biochemical analysis and distinguish between viable and infarcted tissue. Finally, using this method, we showed that pre-exposure to a bacterial endotoxin lipopolysaccharide (LPS) 72 h before hypoxia–ischemia amplifies brain damage in newborn mice, in contrast to its effect of protective preconditioning in adults (Tasaki et al., 1997; Rosenzweig et al., 2004; Eklind et al., 2005). Additional applications of this new vitality-detection method are also discussed.

2. Materials and methods

2.1. Animal surgery

For adult cerebral hypoxia–ischemia (HI), eight-to-twelve week-old male CD1 (Charles River, Wilmington, MA) and Thy1-YFP mice (Jackson Laboratories Stock no. 003782, Bar Harbor, ME) were challenged by transient cerebral HI, performed as described

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previously with minor modifications (Adhami et al., 2006; Shereen et al., 2011). Briefly, animals were anesthetized by intraperitoneal (IP) injection of avertin, and the right common carotid artery was ligated by two releasable (Mule) knots of 4-O silt suture. After carotid ligation, mice were infused with 7.5% O_2 balanced by 92.5% N_2 through a face-mask for 60 min. The body tempeture of mice was maintained between 37.5 and 38.5 °C by Digi-Sense Benchtop RTD connected with a heating lamp and rectal probe (EW-89000-10; Cole Parmer, Vernon Hills, IL). The knots on the common carotid artery were released at the end of hypoxic stress.

For lipopolysaccharide (LPS)-sensititized neonatal cerebral HI, LPS (0.3 mg/kg, Sigma) was IP-injected to 5-day-old CD1 mice at 72 h before cerebral HI, as previously described with minor modifications (Eklind et al., 2005). At postnatal day 8, mice were anesthetized by 2% isoflurane and subjected to permanent ligation of the right common carotid artery. Mouse pups recovered for 1.5 h and were then exposed to hypoxia in glass chambers containing 10% O_2 and 90% N_2 in a waterbacth kept at 37 °C. After hypoxic exposure, mice were returned to dams in the animal care facility. The *in vivo* TTC-labeling procedure was performed at 48 h recovery. These animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. In vivo TTC-labeling method

To disrupt BBB, mannitol (0.5–1.4 M, Sigma) prepared in PBS at a temperature of 37 °C was IP-injected to animals (~0.1 ml/g body weight) for 5–180 min as indicated in the text. Mice were anesthetized with avertin and transcardially perfused of PBS followed by 10 ml of 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma). At 10 min after transcardial TTC perfusion, the brains of animals were removed and placed into 4% paraformaldehyde. Alternatively, the extracted brains can be incubated in warm phosphate-saline buffer (PBS) for 30 min to enhance TTC-staining. For biochemical analysis, brains were removed after TTC-perfusion and divided into the contralateral (the left cortex) and lesion sides (the right cortex) for protein extraction.

2.3. Quantification of brain infarction

The post-fixed brains after *in vivo* TTC-labeling were sectioned into 0.8 mm thick coronal slices with Vibratome (Stoelting, Wood Dale, IL) and photographed for quantification as previously described (Yang et al., 2009). Briefly, digital images of 5 slices in each brain were analyzed using the NIH ImageJ 1.4 software. Brain damage was expressed as the ratio of the infarcted area (white area in the right side) to the area of the undamged, contralateral hemisphere.

2.4. Histology and immunohistochemistry

The fixed brains after *in vivo* TTC-labeling were transferred into graded 30% sucrose solution and frozen in O.C.T. compounds for sectioning at 50 μm-thickness using a sliding microtome (SM2000R, Leica, Wetzlar, Germany). YFP was visualized with an Olympus epifluorescent microscope (BX-51). For immunohitochemistry, the following antibodies were used: mouse anti-HSP70 (SPA810; Stressgen, Victoria, Canada), rabbit anti-DARPP32 (A31656; Chemicon, Temecula, CA), and mouse anti-microtubule-associated protein 2 (MAP2; Sigma, St. Louis, MO). The immunoreactivity was detected sequentially by biotinylated secondary antibodies, the Vectastain ABC kit (Vector Lab, Burlingame, CA), and the diaminobenzidine tetrahydrochloride (DAB) reaction.

2.5. Cell fractionation and immunoblot analyses

The cortex from fresh brain tissue after *in vivo* TTC-labeling was subjected to protein extraction or mitochondrial–cytosol fractionation, as described previously (Adhami et al., 2006). For total protein extraction, three part of cortex (contralateral side with red staining and the ipslateral side with or without red staining) were seperated and homogenized in TLB buffer [20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 25 mmol/L Na-pyrophosphate, 2 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Sigma)]. For mitochondrial–cytosol fractionation, the tissue was homogenized in cold buffer (20 mmol/L HEPES, pH 7.4, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.7% protease inhibitor cocktail, 1 mmol/L Na_3VO_4). The extracted protein samples were processed for immunoblotting and visualized by HRP-reactive chemiluminescence reagents (Amersham Biosciences, Arlington Heights, IL). Primary antibodies were rabbit anti-cytochrome *c* (#4272; Cell Signaling, Danvers, MA), goat anti-AIF (#sc9416; Santa Cruz, Santa Cruz, CA), mouse anti-cytochrome oxidase subunit IV (#A21348; Molecular Probes, Invitrogen, Carlsbad, CA), mouse anti-HSP70, rabbit anti-Bcl-xL (#sc634; Santa Cruz), rabbit anti-caspase-3 (#9662; Cell Signaling) and mouse anti-β-actin (#A544; Sigma).

2.6. Reverse-transcription PCR

The RNA from fresh brain tissues after *in vivo* TTC labeling was extracted using TRIzol reagent (Invitrogen) for RT-PCR analysis, as described previously (Sun et al., 2010). Brifely, total RNA was processed with the high-capcacity cDNA reverse transcription kit (Applied BioSystems, Foster City, CA) to transcript cDNAs. The semi-quantitative PCR of mice *Hsp70*, *Caspase-3*, *Bim-EL*, *Bcl-xL* and *Tspo* cDNAs were detected using the following primers, and the cDNA of a housekeeping gene β-actin was measured in parallel as an internal control: *Hsp70*, 5′-AAGCAGACGCAGACCTTCAC-3′ and 5′-AGATGACCTCCTGGCACTTG-3′; *Caspase-3*, 5′-CTATC-TGGACAGTAGTTACAAAAT-3′ and 5′-CAGTCAGAGCTCCGGCAG-TAG-3′; *Bim-EL*, 5′-CTACCAGATCCCCACTTTTC-3′ and 5′-ACCCTCCT-TGTGTAAGTTTC-3′; *Bcl-xL*, 5′-AGGCAGGCGATGAGTTTGAA-3′ and 5′-TGAAGCGCTCCTGGCCTTTC-3′; *Tspo*, 5′-ATGGGGTATGGC-TCCTACATAGT-3′ and 5′-CCACTGACAAGCAGAAGATCG-3′; and β-actin, 5′-GAAGCACTTGCGGTGCACGAT-3′ and 5′-GAAGCACTTGC-GGTGCACGAT-3′. Reaction products were separated by electrophoresis on a 2% agarose gel. Bands were visualized using an electrophoresis image analysis system (Eastman Kodak Co., Rochester, NY).

2.7. Matrix metalloproteinase (MMP) zymography

MMP-9 and MMP-2 zymogram was performed, as previously described (Yang et al., 2009). Briefly, MMP-9 and MMP-2 in brain extracts was pulled-down using the gelatin Sepharose™ 4B beads (GE Healthcare, Buckinghamshire, UK) and separated by electrophoresis in a poly-acrylamide gel containing 0.15% gelatin. After electrophoresis, gels were washed twice with 2.5% Triton X-100 and incubated in reaction buffer (50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$) at 37 °C overnight. The gel was stained with Coomassie blue and destained to reveal the protease activity. Shown are the inverted image of zymogram gels.