PROBENECID PROTECTS AGAINST CEREBRAL ISCHEMIA/REPERFUSION INJURY BY INHIBITING LYSOSOMAL AND INFLAMMATORY DAMAGE IN RATS

R. WEI, a J. WANG, b Y. XU, a B. YIN, c F. HE, a Y. DU, a G. PENG, a AND B. LUO b*

a The Brain Medical Center and the Collaborative Innovation Center for Brain Science, Department of Neurology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China
b Department of Neurology, The First Affiliated Hospital, Anhui Medical University, Hefei 230022, China
c Department of Neurology, Renmin Hospital, Wuhan University, Wuhan 430000, China

Abstract—Probenecid has been used for decades to treat gout, and recent studies have revealed it is also a specific inhibitor of the pannexin-1 channel. It has been reported that the pannexin-1 channel is involved in ischemic injury. Here, we investigated the neuroprotective effect and the possible mechanisms of action of probenecid in global cerebral ischemia/reperfusion (I/R) injury in rats. Twenty minutes of transient global cerebral I/R injury was induced using the four-vessel occlusion (4-VO) method in male Sprague–Dawley rats. Different doses of probenecid were administered intravenously, intraperitoneally, or by gavage before or after reperfusion. Probenecid via all three routes protected against CA1 neuronal death when given before reperfusion. This protective effect continued when probenecid was given at 2 h after reperfusion, but not at 6 h. Interestingly, the protective effect regained if probenecid was given continuously for 7 days after reperfusion. The release of cathepsin B and overexpression of calpain-1 after reperfusion were inhibited, while the upregulation of Hsp70 was strengthened by probenecid pre-treatment. Furthermore, the activation and proliferation of microglia and astrocytes after I/R injury were suppressed by continuous given for 7 days, but only partly by a single dose at 6 h of reperfusion. Thus, our data indicate that probenecid protects against transient global cerebral I/R injury probably by inhibiting calpain–cathepsin pathway and the inflammatory reaction.

Key words: cerebral ischemia/reperfusion injury, calpain-1, cathepsin B, heat shock protein 70, inflammation, probenecid.

INTRODUCTION

Vascular recanalization therapies such as thrombolysis or mechanical embolus removal are the only effective treatments for acute ischemic stroke (Hacke et al., 2008; Koh et al., 2012). Meanwhile, reperfusion may lead to blood–brain-barrier disruption, hemorrhagic transformation, and massive edema etc, leading to further damage; this is called ischemia/reperfusion (I/R) injury (Molina and Alvarez-Sabin, 2009). The mechanisms underlying I/R injury have been investigated extensively and many studies have sought efficient drugs and molecules to reduce it. But to date, few neuroprotectants have been successfully developed and used in the clinic. Consequently, it is necessary to explore novel therapeutic strategies for I/R injury.

Probenecid is widely used in the clinic, such as gout treatment and an antibiotic synergist. Recently, probenecid was found to be a specific inhibitor of the pannexin-1 (Panx1) channel (Silverman et al., 2008; Ma et al., 2009). Panx1, one of the pannexin family, is broadly expressed, especially in the pyramidal neurons of the central nervous system (Bruzzone et al., 2003; Cone et al., 2013). Panx1 forms non-selective membrane channels that are permeable to ions, nucleotides, and other small molecules (<900 Da), and is involved in ATP release, Ca²⁺-wave propagation, blood flow regulation, and immune responses (Bao et al., 2004; Pelegrin and Surprenant, 2006; Vanden Abeele et al., 2006; Billaud et al., 2011; Penuela et al., 2013). Furthermore, it is known to participate in pathological processes such as inflammation, tumorigenesis, epileptic seizures, and ischemic cell death (Thompson et al., 2006; Kanneganti et al., 2007; Lai et al., 2007; Santiago et al., 2011). A recent report on permanent focal cerebral ischemia demonstrated that infarct size is reduced and neurological outcome improves when pannexins (Panx1 and Panx2) are knocked out in mice (Bargiotas et al., 2011). Therefore, pannexin channels may be a potential pharmacological target for the prevention of I/R injury.

A few studies have shown that probenecid may be an effective neuroprotective drug for ischemic stroke (Nozaki and Beal, 1992; Colin-Gonzalez and Santamaría, 2013; Xiong et al., 2014); however, in most of those studies, the probenecid was given in combination with other...
agents, its definite protective effect is not clearly elucidated. In this study, we investigated the protective effect of probenecid against global cerebral I/R injury, the effects of the route and duration of administration, and the underlying mechanisms in rats.

**EXPERIMENTAL PROCEDURES**

**Animals**

The study protocol was approved by the Medical Faculty Ethics Committee of the Zhejiang University. All experimental procedures were performed in accordance with the Guide for the Care and Use of Experimental Animals. Male Sprague–Dawley rats (280–350 g), from the experimental animal center in the Zhejiang Academy of Medical Sciences, were allowed free access to food and water under a 12/12-h light/dark cycle before experiments.

**Transient global cerebral I/R injury model**

Twenty minutes of global cerebral ischemia was induced by the four-vessel occlusion (4-VO) method established by Pulsinelli, with slight modifications, as routinely used in our laboratory (Wang et al., 2011; Chu et al., 2012). Briefly, anesthesia was induced with 4% (w/v) choral hydrate (400 mg/kg, intraperitoneally (i.p.)), then the bilateral common carotid arteries (CCAs) were freed and both vertebral arteries were permanently electrocauterized. Rats were allowed to recover for 24 h after closing the surgical incisions. On the following day, anesthesia was induced with 4% isoflurane, the surgical incision in the neck was opened and both CCAs were occluded with aneurysm clips for 20 min to induce global cerebral ischemia, then the clips were removed for reperfusion. Rectal temperature was maintained at 37 ± 0.5 °C with a heating pad throughout the procedures, then the rats were moved to an animal incubator in order to maintain proper temperature, until fully awake. Sham-operated rats were subjected to the same procedures as ischemic rats, except for occlusion of the CCAs. Rats that lost the righting reflex, had dilated pupils, and did not have seizures were selected for experiments.

**Drug administration and experimental groups**

Probenecid (Sigma, St. Louis, MO, USA) was dissolved in 2 mM NaOH, and the pH was adjusted to 7.0 with 0.2 M HCl. Then it was diluted with 0.9% NaCl to the needed concentrations (0.1, 1, 2, or 10 mg/ml).

Probenecid was administered through the jugular vein (intravenously (iv)) 10 min before ischemia in a total volume of no more than 0.3 ml. Forty rats were randomly divided into five groups to access neuronal damage (eight per group): sham-operated group (sham), vehicle-treated I/R group (vehicle), and three probenecid-pretreated groups (pre-0.1 mg, pre-1 mg, and pre-10 mg). Another 36 animals divided into three groups (sham, vehicle and pre-1mg group), were decapitated at 1, 2, and 3 days of reperfusion and brain tissues were collected for immunofluorescent staining and western blotting.

A single dose of 2 mg/kg probenecid (at 1 mg/ml) was administered ip at 10 min of ischemia (post-10min group), and at 2 h (post-2h group) and 6 h (post-6h group) of reperfusion. Thirty-two rats were divided into four groups (eight per group): vehicle group and three probenecid-treated groups (post-10min, post-2h and post-6h). To determine whether probenecid has cumulative protective effects, the rats (n = 16, eighth rats per group) were given 2 mg/kg ip at 2 h or 6 h of reperfusion as above, then the same dose was given daily for 6 days (post-2h + 6d group and post-6h + 6d group). Rats for immunofluorescence staining (glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule-1 (Iba-1) examination) in post-2h + 6d group and post-6h + 6d group were the same as HE staining.

To explore the effect of probenecid given by gavage, the same volume of vehicle (vehicle group) or 5 mg/kg probenecid (at 2 mg/ml) was given daily by gavage for 7 days before ischemia (pre-7d group) or started at 2 h of reperfusion (post-2h-7d group) (n = 24, 8 rats per group).

**Determination of neuronal damage**

For neuron counting, rat brains were removed at 7 days of reperfusion and stained with hematoxylin and eosin (HE) procedure as described previously (Wang et al., 2008; Chu et al., 2012). Photomicrographs of the CA1 region were taken using a digital camera (Olympus DP20, Tokyo, Japan) connected to an inverted microscope (Olympus BX51, Tokyo, Japan). The number of surviving neurons per mm length in the CA1 pyramidal cell layer from four sections per animal at the dorsal hippocampal level was counted as neuron density (cell/mm) by a blinded observer.

**Immunofluorescent staining**

Briefly, 4-μm paraffin-embedded coronal sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling the sections in 10 mM citrate buffer (pH 6.0) for 10 min. After washing with phosphate-buffered solution (PBS), sections were incubated with 0.3% H2O2 in PBS for 10 min, blocked with 1% bovine serum albumin containing 0.3% Triton-X 100 for 1 h at room temperature, and incubated overnight at 4 °C with primary anti-cathepsin-B antibody (1:250, Abcam, Cambridge, MA, USA), anti-GFAP antibody (1:200, Abcam, Cambridge, MA, USA), or anti-Iba-1 antibody (1:200, Abcam, Cambridge, MA, USA). Then, the sections were incubated for 2 h in fluorosome-coupled secondary antibody (Alexa 568, Molecular Probes, Eugene, OR).

Images were captured using a confocal laser-scanning microscope (Olympus, FV1000, Japan) at the same microscopic settings. To quantify cathepsin B and glial activation, the integrated optical density (IOD) of immunofluorescence images from the CA1 region was measured using ImageJ software (ImageJ 1.37v; Wayne Rasband). Three high-power images (400× for glial and 800× for cathepsin B) were randomly selected for each
animal, and the mean IOD of these fields was considered the value for that animal.

**Western blotting**

Rat brains were harvested at 1, 2, and 3 days of reperfusion (three rats at each time point), and hippocampal tissues were rapidly isolated on ice and processed for western blot as described previously (Wang et al., 2011). Primary antibodies to calpain-1 (1:2000, Abcam, Cambridge, MA, USA), heat shock protein 70 (Hsp70, 1:2000, Epitomics, Burlingame, California, USA), and β-actin (1:2000, Sigma, St. Louis, MO, USA) were used. Specific bands were detected by enhanced chemiluminescence and images were captured on X-ray film, scanned, and analyzed with ImageJ. The optical density (OD) of the calpain-1 and Hsp70 bands was first divided by the β-actin value of the same well, and the OD ratio was normalized to that of the sham-operated control.

**Statistical analysis**

All data are expressed as the mean ± SD. Statistical analysis was carried out by a one-way analysis of variance (ANOVA) followed by Student-Newman–Keuls tests. *P* < 0.05 was considered significant.

**RESULTS**

**Intravenous probenecid before ischemia attenuates I/R-induced hippocampal CA1 neuronal death in rats**

To assess whether probenecid protects against global cerebral I/R injury, three doses were given intravenously 10 min before ischemia. In the sham group, the number of surviving neurons in the CA1 pyramidal cell layer was 195.9 ± 4.6/mm, while it decreased to 17.5 ± 10.5/mm (8.9% of the sham group) in the vehicle group after 20-min global ischemia and 7 days of reperfusion (Fig. 1A). After probenecid pretreatment, the value increased to 42.2% of the sham group in the pre-0.1mg group, 74.1% in the pre-1mg group, and 56.5% in the pre-10mg group (Fig. 1A, B). These results suggested that iv probenecid pretreatment protected against the hippocampal CA1 neuronal death induced by 20-min global I/R injury, and 1 mg/kg had the highest efficacy. Therefore, 1 mg/kg was used in the following experiments.

**Protective effect of intraperitoneal probenecid after ischemia**

Next, we tested the neuroprotective effects of a single ip dose of probenecid at 10 min of ischemia and at 2 and 6 h of reperfusion. The number of surviving neurons was 62.1% of the sham group (*P* < 0.01, vs. the vehicle group) in the post-10min group, 38.8% (*P* < 0.01, vs. the vehicle group) in the post-2h group, and 19.7% (*P* > 0.05, vs. the vehicle group) in the post-6h group (Fig. 2A, C). These results suggested that the protective effect of ip probenecid continued to 2 h of reperfusion, decreased when given at 2 h of reperfusion, and disappeared when given at 6 h of reperfusion.

Subsequently, to test whether prolonged use improves its neuroprotective effects, probenecid was administered daily for another 6 days. The number of surviving neurons increased slightly in the post-2h+6d group (47.5% of the sham) over the post-2h group (38.8%) but the difference was not significant, while it increased significantly in the post-6h+6d group (43.8%) over the post-6h group (19.7%) (Fig. 2B, C). These data demonstrated that prolonged use of probenecid has cumulative neuroprotective effects even when given after 6 h of reperfusion.

**Probenecid has similar protective effect with intravenous, intraperitoneal, and gavage routes of administration**

To test whether probenecid has protective effects when given by gavage, it was administered by this route for 7 days before or after ischemia. The proportion of surviving neurons was 71.7% of the sham group in the pre-7d group and 46.7% in the post-2h-7d group, both of which were higher than the vehicle group (Fig. 3A, B; *P* < 0.01).

We then analyzed whether there were differences among the routes of administration. When probenecid was given before reperfusion, the protective effect did not differ significantly among the routes (Fig. 3C). Furthermore, when probenecid was given after ischemia, it had time-dependent and route-independent neuroprotective effects (Figs. 2 and 3B, D). These data suggested that the neuroprotective effects of probenecid are route-independent, whether administered before or after ischemia.

**Probenecid pretreatment inhibits I/R-induced upregulation and translocation of cathepsin B in hippocampal neurons**

Cathepsin-B, which is abundant in neurons, is one of the major acidic hydrolases in the lysosome. Many studies have shown its upregulation and translocation from lysosomes into the cytosol after I/R injury, demonstrating a change of lysosomal permeability (Windelborn and Lipton, 2008; Yamashima and Okawa, 2009; Wang et al., 2011). So, we performed immunofluorescence staining to assess the effects of probenecid on the change of cathepsin-B after 20-min global cerebral I/R injury. In sham-operated rats, cathepsin-B staining displayed a granular and lysosomal location in hippocampal CA1 neurons (Fig. 4A). After I/R injury, cathepsin-B granules became progressively larger and irregular from 24 h of reperfusion, after which this was replaced by diffuse cytoplasmic and nuclear staining (Fig. 4B(a,b)). At 72 h of reperfusion, the cathepsin-B staining gradually disappeared with the death of neurons (Fig. 4B(c)). However, after probenecid pretreatment (pre-1mg group), the above changes were observed in only small number of dying neurons with pyknotic nuclei, and lysosomal granular staining was retained in most normal-looking neurons (Fig. 4C). The IOD values for cathepsin-B in the probenecid pretreatment groups were much lower.
than those in the vehicle groups at the same reperfusion times (Fig. 4D, \( P < 0.01 \)).

**Probenecid pretreatment downregulates calpain-1 expression and upregulates Hsp70 expression after cerebral I/R injury**

The above results suggested a change in lysosomal membrane permeability to cathepsin-B. Calpain-1 and Hsp70 are major molecules involved in this process, so their expression was analyzed to indirectly assess the influence of probenecid on lysosomal membrane permeability. The expression of calpain-1 in the hippocampus increased significantly from 24 to 72 h after I/R injury and peaked at 24 h of reperfusion, while this was inhibited by probenecid pretreatment (Fig. 5A, C). The expression of Hsp70 was also upregulated from 24 h to 72 h and peaked at 48 h of reperfusion. On the contrary, its upregulation was potentiated by probenecid pretreatment (Fig. 5B, D).

**Prolonged probenecid treatment after reperfusion reduces the I/R-induced inflammatory reaction**

Our HE results suggested that the protective effect of probenecid regained with prolonged treatment for 7 days, although it disappeared when given as a single dose at 6 h of reperfusion. To investigate the possible mechanisms, changes in astrocytes and microglia were assessed by immunofluorescence, since it has been reported that the inflammatory reaction is a deleterious factor in the late period of I/R injury.

In the sham group, only a few cells with long, thin processes stained positively for GFAP. However, in the vehicle group, robustly increased GFAP immunoreactivity with hypertrophic cellular morphology was observed, suggesting the proliferation and activation of astrocytes (Fig. 6A). Immunostaining for Iba-1 (a specific marker of microglia) showed only a few scattered ramified microglia (resting microglia) in the sham group. After 7 days of reperfusion, the number of microglia increased markedly in the CA1 region, and the resting microglia transformed into ameboid-like cells with plump cell bodies and short, thick processes, indicating they were activated (Fig. 6B). These changes in astrocytes and microglia after I/R injury were significantly inhibited by prolonged probenecid post-treatment (post-6h + 6d group), and were partly inhibited by a single dose of probenecid administered at 6 h of reperfusion (post-6h group) (Fig. 6C, D). These results indicated that probenecid suppresses the inflammatory
reaction induced by global cerebral I/R injury, especially with prolonged treatment.

**DISCUSSION**

Our data demonstrated that a single dose of probenecid given intraperitoneally had neuroprotective effects even after reperfusion, but this weakened with time and disappeared when given at 6h of reperfusion. Surprisingly, the disappeared protection regained if the drug was given ip for 7 days, even if the first dose was given at 6h after reperfusion. Further, there were no significant differences between the post-6h + 6d, post-2h-6d, and post-10min groups, although the protective effect in the post-2h group was somewhat lower than in the post-10min group. These results suggested that probenecid has a cumulative protective effect and thus could be used beyond the 4.5-h stroke thrombolyis window to reduce cerebral I/R injury (of course, the earlier the better). Furthermore, our data showed that probenecid had protective effects whether it was given by gavage for 7 days before or 2h after ischemia, suggesting it can be given orally. Therefore, probenecid can be conveniently delivered by iv or orally without the need for intracerebroventricular administration like many other neuroprotectants, because it can cross the blood-brain-barrier and act on the nervous system directly (Kartzinel et al., 1976; Cowdry et al., 1983). These results indicate that probenecid is a promising neuroprotective drug for clinical application, since it is cheap, readily available, and effective as early as the beginning of ischemia.

In this study, we found that the neuroprotective effect of probenecid is dose-dependent, and 1 mg/kg iv had a better protective effect than 0.1 mg/kg and 10 mg/kg administered before ischemia. Although several studies have shown that probenecid protects against brain injury, in most of these studies the probenecid was combined with other drugs to enhance the accumulation of kynurenic acid (the only known endogenous excitatory amino-acid receptor antagonist in the central nervous system) (Nozaki and Beal, 1992; Robotka et al., 2008; Sas et al., 2008), which in turn is responsible for enhanced inhibition of N-methyl-D-aspartate receptors (NMDARs) and α7 nicotinic acetylcholine receptors (α7nAChRs), thus reducing neuronal excitability and avoiding cascades of toxic intracellular events (Vamos et al., 2009; Colin-Gonzalez and Santamaria, 2013). So the neuroprotective effects of probenecid were considered to be indirect, and the doses (50–200 mg/kg, ip) were much higher than the 2-mg/kg used in our study. However, intraperitoneal probenecid (200 mg/kg) just increased the kynurenic acid levels by 10-fold.
Recent studies indicated that these Ca$^{2+}$ entry mechanisms are significantly facilitated by Panx1 channel activity (Thompson et al., 2008; Orellana et al., 2011; Weilinger et al., 2013), and activation of the Panx1 channel also leads directly to rapid Ca$^{2+}$ overload or the efflux of ATP into the extracellular space (Bao et al., 2004; Vanden Abeele et al., 2006; Reigada et al., 2008; Dvoriantchikova et al., 2012). Extracellular ATP activates purinergic P2 receptors at the cellular surface; they then induce IP3-mediated Ca$^{2+}$ release from the endoplasmic reticulum and downstream signaling cascades that result in neurotoxicity (Chao et al., 2012; Cisneros-Mejorado et al., 2015b). Interestingly, one recent study showed that probenecid also has a direct inhibitory effect on the P2X7 receptor (Bhaskaracharya et al., 2014), thus could blocks ATP-induced calcium influx. Besides, many studies demonstrated that P2X7 receptor and Panx1 could act synergistically (Pelegrin and Surprenant, 2006; Locovei et al., 2007; Gulbransen et al., 2012), and they operate in the same deleterious signaling cascade leading to neuronal and tissue demise (Cisneros-Mejorado et al., 2015a). So, we supposed that probenecid, a specific inhibitor of Panx1 channels, can cross the blood–brain-barrier and thus directly reduce the Ca$^{2+}$ overload in neurons.

Fig. 3. Protective effects of probenecid given by different routes and times. (A) Images of HE-stained hippocampus in vehicle-treated, pre-7d, and post-2h-7d groups (5 mg/kg, by gavage) at 7 days of reperfusion. (B, C, D) Quantification of surviving neurons per mm in the CA1 region. All data expressed as mean ± SD, n = 8. ** P < 0.01 vs. sham; ## P < 0.01 vs. vehicle; ^# P < 0.05 vs. pre-7d in (B). ^# P < 0.01 vs. vehicle; ^P > 0.05, among pre-1.0 mg, pre-7d, and post-10min groups in (C). *P < 0.01 vs. vehicle; ^P < 0.05 vs. post-10 min; ^P > 0.05, among post-2h, post-2h + 6d, and post-2h-7d groups in (D).

Excitotoxicity is one of the major damaging mechanisms in the early stage of ischemic neuronal death, and Ca$^{2+}$ influx via ligand-gated channels such as NMDA and purinergic P2 receptors, is a key factor (Hardingham, 2009; Matute and Cavaliere, 2011).
Fig. 4. Cathepsin-B immunoreactivity after I/R injury and 1 mg/kg probenecid iv. (A) Cathepsin-B staining showed a lysosomal punctate staining pattern in the cytoplasm of CA1 neurons from sham-operated rats; Scale bar = 10 μm. (B) Cathepsin-B granules became progressively larger (increased expression) and irregular after I/R injury (a), and most of the cytoplasm and the nuclei were also cathepsin B-positive (b,c). (C) Increased expression and translocation of cathepsin B was inhibited by probenecid pretreatment (a,b,c). (D) IOD values for cathepsin-B expressed as mean ± SD. *P < 0.01 vs. sham group; #P < 0.05 and ##P < 0.01 between the two indicated groups; no significant differences among sham and 1 mg/kg probenecid pretreatment groups.

Fig. 5. Western blot analysis of calpain-1 and Hsp70 expression. (A,B), Representative western blots of calpain-1 (A) and Hsp70 (B) in the hippocampal region after I/R injury and 1 mg/kg probenecid pretreatment. (C,D) Calpain-1 and Hsp70 expression in the hippocampal region increased significantly from 24 h to 72 h of reperfusion; the calpain-1 expression was inhibited by probenecid pretreatment, but on the contrary, Hsp70 was potentiated. *P < 0.05, **P < 0.01 vs. sham group; #P < 0.05, ##P < 0.01, xP > 0.05 between the indicated groups.
Ca\(^{2+}\)-overload activates many downstream phospholipases and proteases essential for cellular integrity, leading to membrane degradation. Among which, the activation of calpain-1 can cause lysosomal rupture and subsequent release of cathepsins into the cytoplasm in the process of I/R injury. Many studies have found that inhibitors of calpain-1 and cathepsins have good neuroprotective effect against cerebral I/R injury, which lead to the formation of "lysosomal-centric" hypothesis for ischemic brain damage (Tsubokawa et al., 2006; Kilinc et al., 2010; Sahara and Yamashima, 2010; Lipton, 2013; Xu et al., 2014). In this study, we found that probenecid pretreatment strongly inhibited calpain-1 expression and the release of cathepsin B from lysosomes after I/R injury. Hsp70 mainly functions as a chaperone enabling the cell to cope with harmful aggregations of denatured proteins during and following various insults such as heat, ischemia, and other oxidative stresses. It has been reported that the expression of HSP70 increased in the ischemic hippocampus in this 4-VO model and valproic acid treatment resulted in a further increase (Xuan et al., 2012; Kilinc et al., 2010; Sahara and Yamashima, 2010; Lipton, 2013; Xu et al., 2014). In this study, we found that probenecid pretreatment strongly inhibited calpain-1 expression and the release of cathepsin B from lysosomes after I/R injury. Hsp70 mainly functions as a chaperone enabling the cell to cope with harmful aggregations of denatured proteins during and following various insults such as heat, ischemia, and other oxidative stresses. It has been reported that the expression of HSP70 increased in the ischemic hippocampus in this 4-VO model and valproic acid treatment resulted in a further increase (Xuan et al., 2012), and it has also been reported that Hsp70 inhibits injury-induced permeabilization of the lysosomal membrane (Yamashima, 2012). Our results also demonstrated that probenecid enhanced the expression of Hsp70 after I/R injury, an effect that may stabilize the lysosomal membrane. Based on these results, inhibiting the activation of the calpain–cathepsin pathway in the early stages of I/R injury may be one of the protective mechanisms of action of probenecid.

Loss of CA1 neurons after brief global ischemia is a delayed manner, that neurons appear viable for the first hours/days and some physiologic parameters such as cerebral perfusion, glucose metabolism and energy state are measured normal (Mies et al., 1990) though inhibited cerebral protein synthesis (Widmann et al., 1991). Until day 2 postischemic, then energy metabolism begins to deteriorate without major changes in blood flow or recovery of protein synthesis in parallel with the onset of irreversible neuron damage. So, the disappearance of neuroprotection after 6 h posts ischemia most probably accounts for the onset of irreversible damage to most of neurons which may be partially prevented with supplementary treatment dose. Neuroinflammation, which is triggered by cerebral I/R injury and characterized by the activation of microglia and astrocytes, exacerbates neuronal death and contributes to the second stage of I/R injury (Amantea et al., 2009). Therefore, anti-inflammatory therapy may be promising therapeutic strategy for cerebral I/R injury (Shah et al., 2009). Panx1 is an initiating factor and an amplifier of inflammatory signaling, which is associated with secondary cell death, and thus Panx1 could be the prime target for the suppression of inflammation (Locovei et al., 2007; Silverman et al., 2009; Gulbransen et al., 2012; Shestopalov and Slepak, 2014). Xiong reported that probenecid suppresses

---

**Fig. 6.** Probenecid inhibits the inflammatory reaction in the hippocampal CA1 region induced by global cerebral I/R injury at 7 days of reperfusion. (A) Astrocytes became larger and their GFAP immunoreactivity increased markedly in the vehicle group (b), suggesting that I/R injury induced astrocyte activation and proliferation. The changes were partly inhibited in the post-6h group (c) and strongly in the post-6h + 6d group (d). (B) The number of Iba-1-immunoreactive cells (microglia) increased markedly and transformed from resting microglia into ameboid-like cells (activated microglia) in the vehicle group (b); this was partly inhibited in the post-6h group (c) and strongly in the post-6h + 6d group (d) (Scale bars = 20 μm). (C,D) IOD values for GFAP (C) and Iba-1 (D) are expressed as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. sham group; #P < 0.05, ##P < 0.01 vs. vehicle group; &^P < 0.01 vs. post-6h group.
inflammatory processes and reduces the release of high mobility group box-1 (HMGB1, a pro-inflammatory cytokine) from neurons after focal cerebral ischemia in mice, and probenecid has also been reported to reduce injury due to inflammation and infection in acute Pseudomonas aeruginosa pneumonia (Wonnenberg et al., 2014; Xiong et al., 2014). In this study, the activation of glial cells and the proliferation of microglia in the hippocampus were evident at 7 days after I/R injury, and these were strongly inhibited by probenecid given for 7 days after reperfusion, and partly inhibited by a single dose given at the beginning of 6 h of reperfusion. These results indicated that probenecid suppresses the inflammatory response and associated secondary cell death after I/R injury, which may explain the regain of the protective effect when given for 7 days, even when the first dose was given at the beginning of 6 h of reperfusion.

CONCLUSIONS

Our data show that probenecid provides time-dependent and route-independent neuroprotection against neuronal death induced by global cerebral I/R injury probably via inhibition of calpain–cathepsin pathway and the inflammatory response. Because probenecid has long been used in the clinic and no major toxicity has been reported, it is likely to be useful in reducing brain I/R injury. Further studies are needed to confirm the protective effect, determine the optimal dose, reveal the possible mechanisms in other ischemic models, and finally carry out clinical trials.

Acknowledgments—This work was supported by the National Natural Science Foundation of China (81271272 and 81100877), the Ph.D. Programs Foundation of the Ministry of Education of China (2011420120003), Zhejiang Provincial Natural Science Foundation of China (LQ13H090001 and LY13H090004), the Key Research Program for Traditional Chinese Medicine of Zhejiang Province (2013ZZ2010), and the Key Subjects Construction of Traditional Chinese Medicine of Zhejiang Province.

We thank Dr. I.C. Bruce for reading the manuscript.

REFERENCES


(Accepted 28 May 2015)