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Research report

Benzamide protects delayed neuronal death and behavioural impairment in a mouse model of global cerebral ischemia

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ABSTRACT

The present study is aimed at evaluating the functional and neuroprotective effect of benzamide, a poly-(ADP-ribose) polymerase (PARP) inhibitor on delayed neuronal death (DND) in hippocampus CA1 region and memory impairment following global cerebral ischemia (GCI) in a mouse model. GCI was induced by bilateral common carotid artery occlusion (BCAo) for 20 min followed by reperfusion for 9 days. Postischemic continuous treatment with benzamide (160 mg/kg b w i.p. for 9 days) significantly reversed the GCI-induced anterograde memory impairment in passive avoidance step through and elevated plus maze tasks. The observed memory impairment in vehicle treated ischemia group was found to be well correlated with DND and downregulation of cholinergic muscarinic receptor-1 expression, which was possibly mediated by inflammation and apoptosis, as revealed from inducible nitric oxide synthase (iNOS) expression and number of TUNEL positive neurons in hippocampus CA1 region. It is clear from the present experiment that benzamide treatment significantly decreases the iNOS expression and number of apoptotic neurons and thereby improves the neuronal survival and memory during GCI. Our present findings provide compelling evidence that multiple doses of benzamide treatment is a promising therapeutic approach for cerebrovascular and neurodegenerative diseases, which deserves further clinical evaluation.

1. Introduction

Global cerebral ischemia (GCI) induces delayed neuronal death (DND) [25] along with degeneration of cholinergic neurons in hippocampus CA1 region after 2–3 days of reperfusion [22]. Cholinergic and glutamatergic neurons in hippocampus are crucially involved in learning and memory. Dysfunction in any one or both of these systems leads to severe form of dementia [1,7]. Clinical as well as experimental reports reveal anterograde memory impairment as a consequence of GCI [4,37].

Cerebral ischemia induces enhanced free radical [NO•, $ONOO^-$, $•^-O_2$, and •OH] formation, which leads to DNA strand break thereby activating poly-(ADP-ribose) polymerase (PARP), an enzyme involved in maintaining genomic DNA integrity [36,44,48]. PARP is a multifaceted enzyme involved in various cytotoxic mechanisms like inflammation, mitochondrial dysfunction, necrosis and apoptosis. It is noteworthy that all these cell death machineries are well recognized in GCI-induced DND [19,45,49,50]. This suggests that PARP plays a detrimental role in ischemic neuronal death, which deserves in-depth investigation.

Emerging body of reports suggest that PARP knockout mice are resistant to focal cerebral ischemia [13]. Although the PARP inhibitor attenuates the energy crisis during GCI in short-term experiments [26,39], it fails to protect neurodegeneration as well as apoptotic neuronal death after ischemia [32,34,46]. In addition, PARP inhibitor was found to aggravate neuronal death in the rat model of GCI [35]. It is very important to note that all of these fore mentioned experiments followed only a single dose administration of PARP inhibitors during pre/postischemia. However, Strosznajder et al. reported that PARP activity consistently increases up to 7 days postischemia in acute GCI [48]. Moreover, the important characteristic hippocampal CA 1 neurodegeneration occurs in a delayed manner during GCI. These reports suggest that GCI-induced prolonged over activation of PARP may contribute to DND, which was well correlated with the PARP activation in cerebral ischemia [23].

Benzamide, a potent PARP inhibitor was found to mitigate the NAD^+ depletion in MPTP, methamphetamine-induced neurodegeneration as well as kainate and NMDA-induced excitotoxic lesion in a mouse model. Benzamide administration (160 mg/kg i.p.) does not induce hypothermia and reaches the CNS in 30 min in the concentration range of 0.09–0.64 mM, at which, it shows neuroprotection [9–11]. Further, the *in vitro* experiments showed that glutamate-induced excitotoxicity in cultured cerebellar granule cells is efficiently prevented by benzamide [12]. In addition, ben-

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zamide significantly attenuated apoptosis in rat cortical neurons by decreasing the mitochondrial permeability transition, release of cytochrome *c*, apoptosis inducing factor (AIF) and endonucleases G from mitochondria during oxygen glucose deprivation induced *in vitro* ischemia [50].

Based on these evidences a hypothesis has been drawn that the sustained inhibition of PARP in the early as well as late phase of GCI is critical for a better neuroprotection. Hence, in the present experimental study an effort has been made to evaluate the effect of long-term benzamide treatment on transient cerebral ischemia-induced memory impairment and neuronal death.

2. Materials and methods

Benzamide, Hoechst 33342 (Fluka), Tris buffer, Triton X 100, EGTA, benzamidine HCl, aprotinin, leupeptin, pepstatin, SDS, paraformaldehyde (Sigma–Aldrich, USA), Apo-BrdU-IHC *in situ* DNA Fragmentation Assay Kit (BioVision, Inc., USA), hematoxylin and eosin (Merck, India), polyclonal rabbit-cholinergic muscarinic receptor-1 (Santa-cruz, USA), inducible nitric oxide synthase (iNOS) antimouse antibody (Pharmingen, BD Biosciences, USA), Goat antirabbit HRP conjugated and goat antimouse FITC labeled antibody (Santa Cruz Biotechnology, CA, USA). All chemicals were purchased from Sigma–Aldrich, USA unless otherwise specified.

2.1. Groups

Male Balb/c mice weighing 30–35 g from the in-house animal facility were used in this experiment. The animals were maintained at 27 ± 2 °C for a 12-h dark and light cycle. Animals had free access to food and tap water. All the experimental procedures were performed according to the guidelines for the Care and Use of Laboratory Animals approved by CPCSEA and Institutional Animal Ethics Committee.

A total of 48 animals were segregated into four groups (n = 12/group). Group I (benzamide + GCI), Group II (vehicle + GCI), Group III (benzamide + sham operated), Group IV (sham operated). The mice were treated with benzamide (160 mg/kg) [34] within 5 min after ischemia or saline as vehicle 10 ml/kg by i.p. injection daily at 9 a.m. for 9 days (until sacrifice).

2.2. Induction of GCI/reperfusion injury

In the present study we used Balb/c mice for the induction of GCI, since it has an incomplete circle of willis similar to gerbils and C57BL/6J (posterior communicating artery is either poorly developed or absent) [3,29]. The transient GCI was induced by occluding both the common carotid arteries [58] with microclips for 20 min under ketamine (100 mg/kg i.p.) and xylazine (2 mg/kg i.p.) anesthesia on a temperature maintained platform at 37 ± 0.5 °C to prevent hypothermia. The microclips were removed after 20 min and reperfusion was allowed for 9 days. Animals that were assigned to the sham operated group also underwent the same surgical procedure, but without ligation of arteries. As postsurgical care, all the animals were maintained in individual cages and placed in a temperature controlled chamber. Postischemia, out of 24 animals in Groups I and II, a total of three animals died due to respiratory arrest within 48 h. Additionally, one animal in Group II showed severe convulsions along with squatting posture after 24 h postischemia. All these animals were excluded from the study and replaced. A 2% xylocaine (AstraZeneca, India) and povidone-iodine solution IP (Wockhardt, India) were applied topically on surgical wounds.

2.3. Behavioural studies

To study the effect of benzamide on learning and memory alterations during cerebral ischemia, passive avoidance (PA) step through and elevated plus maze (EPM) tasks were performed on day 1 (acquisition trial), days 2 and 9 (retention trial) postischemia/surgery. All the behavioural experiments were performed between 8 a.m. and 2 p.m. All the groups of animals were exposed to behavioural tasks after an hour of drug/vehicle administration. The neurological functions were evaluated by climbing test and beam-walking test as described earlier [14,16].

2.3.1. Elevated plus maze

Elevated plus-maze is a well-established task to evaluate spatial memory in rats and mice [40]. The elevated plus maze consists of two open (16 cm × 5 cm) and enclosed arms (16 cm × 5 cm × 12 cm) extending from a central hub (5 cm × 5 cm), elevated to a height of about 25 cm from the floor. In the acquisition trial, each mouse was placed at the end of an open arm and the time taken to reach the enclosed arm was recorded as transfer latency (TL in seconds). If the animal failed to find the enclosed arm spontaneously on acquisition trial, the TL was recorded as 180 s and the animal was gently guided into the enclosed arm. The animal was allowed to explore the maze for 15 s. Memory retention trial was performed on days 2 and 9 after the acquisition trial [43].

2.3.2. Passive avoidance step-through task

The passive avoidance step-through task is used to measure associative memory. It consists of a 25 cm long box partitioned into light (9 cm × 9 cm × 14 cm) and dark (14 cm × 9 cm × 14 cm) compartments with a grid floor. The light compartment was illuminated with a 60 W bulb, positioned 80 cm above the apparatus. During the acquisition trial, the animal was allowed to explore the light chamber for 5 s. Following this, the guillotine door was opened and time taken to enter the dark chamber was recorded as step-through latency (STL in seconds) and the animal received a 0.2 mA (50 Hz) inescapable electric shock for 2 s. After the acquisition trial, memory retention was tested on days 2 and 9 without the electric shock with a maximum cut off time of 300 s [8].

2.4. Total RNA isolation and PCR amplification

At the end of behavioural experiments six animals from each group were sacrificed under euthanasia and their brains were harvested to dissect hippocampus as described [17] in a cold room maintained at 10 °C. One half of the dissected hippocampus was used for total RNA isolation and the other half for protein isolation.

Total RNA was isolated from hippocampus using TRI reagent (Ambion Inc.) as per the manufacturer's instructions and quantified using NanoDrop (ND-1000 UV–vis Spectrophotometer) at 260 nm. The quality of RNA was verified by analyzing the 260: 280 ratios. The cDNA was synthesized using 5 μg of total RNA by First strand cDNA synthesis kit (Fermentas) as per the manufacturer's instructions. The synthesized cDNA was used for the reverse transcriptase PCR amplification of cholinergic muscarinic receptor-1 (ChM-1r) and iNOS employing a set of specific primers: GAPDH (FP-5′-TTC ACC ACC ATG GAG AAG GC-3′, RP-5′-GGC ATG GAC TGT GGT CAT GA-3′) (Tm 57 °C/30 cycles), iNOS (FP-5′-ATG GAC CAG TAT AAG GCA AG-3′, RP-5′-CTC TGG ATG AGC CTA TAT TG-3′) (Tm 59.1 °C/33 cycles) and ChM-1r Primer (FP-5′-CTG GAA AGA AGA AGA GGA AGA-3′, RP- 5′-GCT GCC TTC TTC TCC TTG AC-3′) (Tm 56 °C/36 cycles) using DNA polymerase (Biotools, Inc.). Densitometry analysis and relative quantities of GAPDH (236 bp) and iNOS (428 bp), ChM-1r (272 bp) PCR products were analyzed using Gene Snap software (Gene Tools, Syngene, MD, USA).

2.5. Western blot

The hippocampus was homogenized in ice-cold RIPA buffer containing a cocktail of protease inhibitors. The total protein content in the homogenate was quantified using Bradford's method. The homogenate equivalent to 30 μg of total protein was resolved on 10% SDS-PAGE, and the proteins were electro transferred to nitrocellulose membrane and blocked with 5% BSA. The membrane was incubated with primary (rabbit polyclonal IgG cholinergic muscarinic recptor-1; 1:5000) and HRP-conjugated secondary goat antirabbit antibodies (1:7500) (Santa Cruz Biotechnology, CA, USA). Blots were then developed by using 3′3-diaminobenzidine; captured and analyzed using Gene Snap software (Gene Tools, Syngene, MD, USA). Reverse transcriptase PCR (RT PCR) and western blot results were expressed as relative mean intensity of the experimental group to their respective controls.

2.6. Histopathology and Immunofluorescence

For Immunofluorescence and histopathology studies six animals per group were anesthetized and perfused intracardially with heparinized 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and kept in a solution of 30% sucrose and 10% glycerol in PBS until use. Thin coronal sections of 5 μm thickness were made using a cryotome (at −20 °C) (CM1850; Leica, Heidelberg, Germany), thaw mounted on a gelatin-coated glass slide and stored at −80 °C until used. The brain sections were stained with hematoxylin and eosin (H and E) for histopathology, TUNEL assay and the alternate sections were used for immunofluorescence study. The sections were permeabilized with 0.1% Triton X 100 in PBS pH 7.4 and then blocked with BSA 5% in PBS pH 7.4 for 1 h at 37 °C, followed by overnight incubation with antimouse iNOS (1:200) antibody (Pharmingen, BD Biosciences, USA). Then the sections were washed thrice with PBS and incubated with FITC labeled goat antimouse antibody (1:500) (Santa Cruz Biotechnology, CA, USA) for an hour in a dark room. The sections were washed with PBS and finally counterstained with Hoechst 33342. After a brief washing the sections were mounted with glycerol/PBS (9:1). The iNOS immunofluorescence and hematoxylin and eosin staining of hippocampus CA1 region (five sections per animal) was observed using Nikon Eclipse E600 with Nikon Y-FL Epifluorescence attachment (Tokyo, Japan). The images were acquired with Evolution VF camera. The fluorescence intensity of the captured images were analyzed with Image-Pro Plus Version 5.1.2 software (Mediacybernetics) and imported into Adobe Photoshop 7.0, for the adjustment of brightness and contrast.

2.7. Apoptotic neuronal death detection by TUNEL method

To identify the apoptotic neuronal death in the hippocampal CA1 region, terminal deoxynucleotidyl transferasemediated biotinylated dUTP nick end-labeling (TUNEL) staining was performed on brain sections with the Apo-BrdU-IHC *in situ* DNA Fragmentation Assay Kit, following the manufacture's instruction (BioVision, Inc., USA) and developed with 3′3-diaminobenzidine in the presence of H_2O_2/urea tablet provided in the kit. After TUNEL reaction, the sections were counterstained