Original Article

Possible mechanism of sustained 5-adenosine monophosphate-activated protein kinase activator associated with neuronal nitric oxide synthase inhibition in global cerebral ischemia-induced neurodegeneration in rats

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ABSTRACT

Global cerebral ischemia (GCI) is a clinical condition that causes a deprivation of blood supply and energy in the brain due to blockade of carotid arteries. The decreased level of oxygen and glucose causes various cellular changes leading to excitotoxicity and oxidative damage. Cerebral ischemia leads to cell death in CA1 region of the hippocampus, which occurs 3–4 days after an initial ischemic insult and neurodegeneration is evident by 3 days of reperfusion, and neuronal death culminates by 6 days. GCI was induced by bilateral carotid artery occlusion for 10 min. 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (50 µg and 100 µg) and 3-Br-7-nitroindazole (10 µg, 20 µg, and 50 µg) were administered once a daily for 4 days as a treatment. Cerebral ischemia leads to 5-adenosine monophosphate-activated protein kinase (AMPK) activation, and further administration of AICAR leads to sustained activation of AMPK. Literature has suggested that AMPK activation causes stimulation of mitochondrial biogenesis resulting in an increase in free radical accumulation. In the present study, cerebral ischemia has been noted to cause: Increased malondialdehyde levels, decreased glutathione levels, increased lactate dehydrogenase levels, and decreased acetylcholinesterase levels, and increased nitrate levels in brain homogenate. AMPK activation through AICAR administration was observed to damage cortex and hippocampal regions of brain as are evident from photomicrographs. Thus, it may be concluded from the present study that AMPK activation in ischemic animals leads to neurodegeneration.

Keywords: Global cerebral ischemia, 5-adenosine monophosphate-activated protein kinase, 5-aminoimidazole-4-carboxamide ribonucleotide, excitotoxicity, hippocampus, neurodegeneration

INTRODUCTION

Cerebral ischemia is a neurodegenerative disease associated with degeneration and death of neurons of the affected brain tissues.[1] Cerebral ischemic stroke is an acute cerebrovascular disease which occurs as a result of sudden interruption of blood supply to a part of brain either due to thrombus formation,[2] embolus occlusion or due to hemorrhage of blood vessels.[3] Cerebral ischemia is characterized by sudden confusion, troubled speaking, troubled walking, dizziness, loss of balance or coordination, sudden severe headache, and moreover loss of memory. Due to interruption due to either of the above-mentioned factors, the blood flow becomes insufficient to meet the metabolic demands of brain tissue.[4] Cerebral ischemia is of two type such as global cerebral ischemia (GCI) and focal cerebral ischemia. In GCI, the reduction of blood flow involves the whole brain or forebrain. Experimental models of global ischemia simulate hypoxic/ischemic state such as that occurring during cardiac arrest, severe hypotension,

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or drowning. In focal cerebral ischemia, the disturbance in blood flow occurs in a localized area.[5] In the commonly used focal cerebral ischemia animal models, the middle cerebral artery (MCA) is occluded either transiently or permanently, which simulates the stroke type of thrombotic occlusion of MCA commonly seen in stroke patients.[6]

10 min of forebrain ischemia and then reperfusion in rat result in a significant increase in the levels of malondialdehyde (MDA), nitrite, and lipid peroxides in the cortex, striatum, and hippocampus region and thus proceed the onset of neuronal death.[7] Cerebral ischemia leads to hippocampal CA1 neuronal death which usually occurs 3–4 days after an initial ischemic insult.[8] Such a delay is essential for the mechanism of this type of cell death. The rodent model of transient forebrain ischemia (bilateral carotid artery occlusion [BCAO]) is characterized by delayed hippocampal CA1 neuronal death.[9] Neurodegeneration is evident by the 3rd day of reperfusion and neuronal death culminates by the 6th day.[8] Furthermore, the hippocampus is associated with learning and memory and one of the most severely affected structures in ischemia.[10] Hence, cerebral ischemia ultimately leads to memory and cognitive impairments. Deficits in learning and memory have also been reported in rats subjected to global ischemia.[11] Moreover, patients having hypoxic/ischemic hippocampal damage are also reported to be amnesic. Ischemic rats are impaired in tasks requiring working and spatial memory such as T-maze, radial arm maze, and Morris water maze.[12] According to a survey by the American Heart Association, cerebral ischemic stroke has been reported as the third leading cause of death. Various drugs have been used for treatment and prevention of cerebral ischemia such as glutamate antagonists, gamma-aminobutyric acid (GABA) agonists, voltage-sensitive calcium channel antagonists, voltage-dependent K^+ channel agonists, Na+ channel antagonists, and free radical scavengers. However, there are various side effects associated with them and no specific agent is considered to be suitable. Hence, it becomes necessary to find out a drug that can be used safely to prevent cerebral ischemia-induced neurodegeneration.

Many studies have suggested 5-adenosine monophosphate-activated protein kinase-activated protein kinase (AMPK) to be a potential target for prevention and treatment of cerebral ischemia-induced neurodegeneration.[13] AMPK is a heterotrimeric serine-threonine kinase, comprising a catalytic α-subunit and regulatory β- and γ-subunits.[14] AMPK is a metabolic energy sensitive kinase and gets activated in the conditions such as hypoxia, ischemia, hypoglycemia, or any other stimuli that may cause energy depletion[13] such as cerebral ischemia or ischemic stroke. However, unlike most peripheral tissue, neurons are exquisitely sensitive to even brief periods of ischemia, because neurons lack the enzymatic machinery necessary for effective glycolysis.[15] Hence, cerebral ischemia leads to deletion of energy, that is, adenosine triphosphate (ATP)/AMP in neurons. Under these conditions, AMPK may get activated by two pathways either the increased AMP binds and phosphorylates Thr-172 residue of α-subunit of AMPK, resulting in activation of AMPK[16] or the increased intracellular Ca^{2+} phosphorylates and activates Ca^{2+}-calmodulin-dependent protein kinase kinase-beta, an upstream activator of AMPK.[17] Once AMPK gets activated, it tries to conserve energy by inhibiting the anabolic processes such as fatty acid synthesis,[18] protein synthesis,[19] and glycogen synthesis, stimulating the catabolic processes such as fatty acid oxidation, glycolysis through activation of phosphofructokinase 2, and glucose uptake.[20] Culmsee *et al.*, 2001, have reported that AMPK activation in cerebral ischemia is neuroprotective possibly through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A, or activation of Raf-1.[21] Moreover, it has also been reported that AMPK activation is neuroprotective through activation of the GABA-B receptor.[14] In contrast to the above findings, it has also been reported that AMPK activation in cerebral ischemia is neurodegenerative possibly through neuronal nitric-oxide synthase (nNOS) activation and $ONOO^-$ formation,[13] activation of Jun N-terminal kinase (JNK) or through inhibition of Akt an enzyme involved in cell growth.[22] Hence, based on these reports regarding the controversial role of AMPK in GCI the present study was designed to find out the role of AMPK in cerebral ischemia-induced neurodegeneration in rats and to find out the possible role of nNOS in mediating AMPK-induced neurodegeneration.

MATERIALS AND METHODS

Experimental animals

Wistar rats, bred in the Central Animal House facility of ISF College of Pharmacy, Punjab, India, and weighing between 150 and 220 g were used. Animals were acclimatized to laboratory conditions before experimentation. The animals were kept in groups of three, in plastic cages with soft bedding, under standard conditions of light and dark cycle, and with free access to food and water. All the experiments were carried out between 08.00 and 17.00 h. The protocol was approved by the Institutional Animal Ethics Committee and carried out in accordance with the Indian National Science Academy guidelines for the use and care of animals.

Drugs and chemicals

Unless otherwise specified, all the chemicals and biochemical reagents of analytical reagent grade and highest purity were used. The following agents were used in the present study: AMPK activator: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Prolab marketing, India) was dissolved in saline (adjusted pH 7.4 with NaOH) and administered intracerebroventricularly (ICV), nNOS inhibitor: 3-bromo-7-nitroindazole (NI) (Prolab marketing, India) was dissolved in 2.5% dimethylsulfoxide (DMSO) and was administered ICV. Rat lactate dehydrogenase (LDH) assay kits (reckon diagnostic) were used.

Treatment schedule

To investigate the effect of AMPK in BCAO induced cerebral ischemia, animals were randomly divided into 12 groups, and each group consisted of 6 animals. All the animals were subjected to training in Morris water maze for 3 days. On day 4, cerebral ischemia was induced using BCAO model. Treatment was started from day 4 and given up to day 7. Group 1, sham control group, was subjected to surgery only but no ischemia was induced; Group 2 received 2.5% DMSO only for 4 consecutive days; Group 3 received AICAR (25 μg/rat, ICV) once daily for a period of 4 days; Group 4 received AICAR (50 μg/rat, ICV) once daily for a period of 4 days; Group 5 received AICAR (100 μg/rat, ICV) once daily for a period of 4 days; Group 6 received AICAR (50 μg/rat, ICV, per se) once daily for a period of

4 days without induction of cerebral ischemia, Group 7 received AICAR (100 µg/rat, ICV, *per* se) once daily for a period of 4 days without induction of cerebral ischemia; Group 8 received 3-Br-7-NI (10 µg/rat, ICV) once daily for a period of 4 days; Group 9 received 3-Br-7-NI (20 µg/rat, ICV) once daily for a period of 4 days; Group 10 received 3-Br-7-NI (20 µg/rat, ICV, *per se*) once daily for a period of 4 days without induction of cerebral ischemia; Group 11 received both AICAR (50 µg/rat, ICV) and 3-Br-7-NI (10 µg/rat, ICV) once daily for a period of 4 days; and Group 12 received AICAR (50 µg/rat, ICV) and 3-Br-7-NI (20 µg/rat, ICV) once daily for a period of 4 days.

Experimental models

Bilateral common carotid artery occlusion (BCCAO) coupled with hypotension

GCI is a clinical outcome occurring as a consequence of conditions such as cardiac arrest and coronary bypass surgery. These conditions cause a deprivation of blood supply and energy in the brain due to blockade of carotid arteries. Approximately 10–20% of cardiac patients show recovery. Others die or persist in a vegetative state. Patients who recover exhibits neurophysiological deficits.[23] Experimentally, global ischemic stroke can be induced by occluding bilateral carotid arteries along with hypotension. It is a simple method and permits rapid screening. It produces delayed and selective neuronal death. Monitoring of physiological variables can be carried out easily. It has been reported that there are no changes in the energy state in the tissue following the only ligation of the carotid arteries, but when combined with systemic hypotension causes severe changes in the energy states. In this model when ischemia was longer, the damage was also seen in the caudoputamen and pars reticulata of the substantia nigra. The major disadvantage of this model is the alteration in the physiological variables as a result of hypotension.[9]

Cerebral ischemia is known to produce severe histopathological damage and related behavior deficits, some of which continue to progress. The model has received considerable attention for its experimental outcome due to its close proximity with human cognitive deficits.

Surgical procedure for the induction of GCI

Transient forebrain ischemia was produced in rats as described by Smith *et al.* In brief, ischemia was achieved by occluding BCCA for 10 min combined with hypotension caused by blood withdrawal and followed by reperfusion. Animals were anesthetized with ketamine (75 mg/kg, i.p.,) and chlorpromazine (3mg/kg, s.c.). The jugular vein and tail artery were catheterized for monitoring blood pressure and for withdrawal of blood. Anticoagulation was achieved by intravenous heparin (200 IU/kg) administration. A transverse incision was made on the skin in the neck, followed by removal of subcutaneous tissue. A vertical incision was made in the muscles surrounding the trachea to expose the right and left CCA and isolate them with a 3–0 silk thread. After the blood circulation was stabilized, the blood pressure was reduced to 50 mm of Hg by the drainage of 1 ml of blood from a tail artery. Ischemia was induced by affixing a clip to each CCA and obliterating the blood flow for 10 min. During this period, environmental temperature was adjusted to 30–37°C by means of an infrared heating lamp, to prevent decay of intraischemic brain temperature. Circulation was restored after 10 min ischemia by removing clips. The blood pressure was restored by re-infusing the shed blood. To minimize systemic acidosis, the animal was administered intravenously 0.5–1 ml of sodium bicarbonate (50 mg/kg). The incisions were closed following removal of all the tubes. The rats were allowed to recover from anesthesia and returned to their cages. During the post-operative period, special care was taken to provide food and water inside the cage of the rat. The animals were assigned to the experimental groups described below, immediately after completion of forebrain ischemia process. The treatment was continued for 4 days.[24,25]

Surgical procedure for ICV administration of drugs

Surgery was performed according to a protocol previously described by Kumar *et al.* Animals were anesthetized with a combination of ketamine (75 mg/kg, i.p.) and chlorpromazine (3 mg/kg, s.c) and placed in a stereotaxic apparatus. The head was positioned in a frame, and a midline sagittal incision was made in the scalp. Single hole was drilled through the skull for placement of injection cannula into the lateral cerebral ventricles. The scalp was then closed with sutures. After surgery, animals received gentamicin (5 mg/kg, i.p.) injection to prevent sepsis. Animals were infused ICV with either saline or AICAR dissolved in saline using a Hamilton microsyringe positioned in the injection cannula. In sham-operated rats, the surgery was identical except for drilling of holes and placement of the cannula. Coordinates for the injection were 1.8 mm posterior to bregma, 0.5 mm lateral to midsaggital line, and 3.6 mm beneath the cortical surface. To promote diffusion, the microsyringe was left in place for a period of 2 min following injection. Special care was taken during the post-operative period to provide food and water inside the cage of the rat.[26] Drugs were administered to different animals according to the grouping of experimental animals given below from day of GCI induction, and the treatment was continued for 4 days.

Grouping of animals

1. Sham control
2. GCI control
3. GCI+AICAR (25 µg/rat, ICV)
4. GCI+AICAR (50 µg/rat, ICV)
5. GCI+AICAR (100 µg/rat ICV)
6. GCI+3-Br-7-NI (10 µg/rat ICV)
7. GCI+3-Br-7-NI (20 µg/rat ICV)
8. 3-Br-7-NI (*per se*, 20 µg/rat ICV)
9. AICAR(*per se*, 100 µg/rat ICV)
10. AICAR (*per se*, 50 µg/rat ICV)
11. GCI+AICAR (50 µg/rat ICV)+3-Br-7-NI (20 µg/rat ICV)
12. GCI+AICAR (50 µg/rat ICV)+3-Br-7-NI (*per se*, 10 µg/rat ICV).

Biochemical parameters evaluated

In the GCI model, all the biochemical evaluations were carried out on the 7th day after induction of ischemia.

Tissue preparation

Animals were sacrificed by decapitation and brains were removed and rinsed with ice cold isotonic saline. Brain tissue samples were

then homogenized with ice cold 0.1 M phosphate buffer (pH 7.4, 10 times w/v of the weight of the tissue). The homogenates were centrifuged at 10,000× *g* for 15 min, and an aliquot of supernatants was separated and used for biochemical estimations.[26]

Measurement of MDA

MDA is a naturally occurring product of lipid peroxidation. The latter is a well-established mechanism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues. The measurement of MDA, which is most abundant of lipid peroxidation products, is a convenient and sensitive method for quantitative estimation of lipid peroxide concentration in many types of samples including drugs, food products, and biological tissues from humans and animals.

The most common method of measuring MDA is based on its reaction with thiobarbituric acid (TBA). The TBA reactive substances assay is a colorimetric method used for the detection of lipid peroxidation in biological materials. MDA reacts with TBA at high temperature (90–100°C) and in acidic conditions. The reaction yields a pink MDA-TBA adduct. This colored complex can be extracted into organic solvents such as butanol and measured by fluorometry or spectrophotometery at 532 nm. MDA measurement was performed by the method of Wills *et al.,* 1965. Homogenate and 0.1 M Tris HCl (pH 7.4) mixture was incubated at 37°C for 2 h. Then, ice cold trichloroacetic acid (10% w/v) was added, and the mixture was centrifuged at 1000× *g* for 10 min. To an aliquot of the supernatant, 0.67% w/v TBA was added and the tubes kept in boiling water bath for 10 min. 1 ml double distilled water was added after cooling the tubes at room temperature, and absorbance was measured spectrophotometrically at 532 nm.

Estimation of glutathione (GSH)

In 1959, Ellman introduced 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) also known as DTNB, as a versatile, water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls. Consequently, Ellman's reagent has proven to be useful as a sulfhydryl assay reagent due to its specificity for -SH groups at neutral pH, high molar extinction coefficient, and short reaction time. GSH is a tripeptide of glycine, glutamic acid, and cysteine. In the red blood cell, the reduced form of GSH is vital in maintaining hemoglobin in a reduced state and hence protecting the cells from oxidative damage. GSH is involved in detoxification of hydrogen peroxide through GSH oxidase. Low levels of GSH are found in deficiencies of key enzymes involved in GSH metabolisms, such as glucose-6-phosphate dehydrogenase, GSH synthase, and GSH reductase. GSH assay is designed to accurately measure reduced GSH in biological samples. The DTNB method combines deproteination and detection into one reagent. DTNB reacts with reduced GSH to form a yellow product. The optical density, measured at 412 nm, is directly proportional to GSH concentration in the sample. GSH was estimated by Ellman's method (1961). Mixture of homogenate and 4% sulfosalicylic acids were kept at 4°C for 1 h. Then, after centrifugation at 1200×g for 15 min at 4°C, the supernatant, phosphate buffer (0.1 M and pH 8), and DTNB (0.4 % w/v in 0.1 M phosphate buffer pH 8) were added to a test tube, and absorbance was measured immediately spectrophotometrically at 412 nm.

Measurement of nitrite/nitrate content

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), is determined in a colorimetric assay based on Greiss reagent according to Green *et al.,* 1982. Measurement of nitric oxide in biological systems needs careful considerations. Nitric oxide is rapidly oxidized to nitrite and/or nitrate by oxygen. The half-life of nitric oxide in a biological matrix is very short, ranging from <1 s in the presence of hemoglobin to ~30 s. Nitric oxide can be measured *in situ* using fast-response amperometric electrode sensors. Alternatively, integrated nitric oxide production can be estimated from determining the concentrations of nitrite and nitrate end products. The measurement of nitrate/nitrite concentration (NOx) or total nitrate and NOx is routinely used as an index of NO production.

Nitric oxide production was determined in the supernatant based on Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid). A mixture of the equal volume of the supernatant and Greiss reagent were incubated at room temperature for 10 min in the dark. Absorbance was measured spectrophotometrically at 540 nm.

Measurement of acetylcholinesterase (AChE)

AChE, also known as RBC cholinesterase, is found primarily in the blood and neural synapses. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation. AChE is a marker of extensive loss of cholinergic system in the forebrain. AChE Assay is based on improved Ellman method, 1961, in which thiocholine produced by the action of AChE forms a yellow color with DTNB. The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample.

The assay mixture contained 0.05 ml of the supernatant, 3 ml of 0.01 M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide, and 0.10 ml of DTNB (Ellman reagent). The change in absorbance was measured immediately at 412 nm using a spectrophotometer.

Measurement of LDH activity

LDH is an oxidoreductase enzyme which catalyzes the interconversion of lactate and pyruvate. When disease or injury affects tissues, the cells release LDH, where it is identified in higher than normal levels. Therefore, LDH is most often measured to evaluate the presence of tissue or cell damage. The non-radioactive colorimetric LDH assay is based on the reduction of the tetrazolium salt MTT in a NADH-coupled enzymatic reaction to a reduced form of MTT which exhibits absorption maxima at 565 nm. The intensity of the purple color formed is directly proportional to the enzyme activity.

Procedure

The level of LDH represents the necrotic cell death, and the level of LDH is increased in neuroinflammation. Measurement of LDH was accomplished using a commercial kit (Reckon Diagnostics, Baroda).

Behavioral study

Morris water maze (MWM)

The maze consisted of white fiberglass circular pool (2m diameter), filled with an opaque mixture of water and white non-toxic dye maintained at 26–28°C. The pool was located in the center of a sound attenuated room. The pool was divided into four equal quadrants: Northeast, southeast, northwest, and southwest. A hidden platform (9 cm dia) submerged 1 cm below the surface of the water was placed in the middle of one of the quadrants.

During the acquisition phase of the experiment, each rat was given four trials each day until they took approximately 15 s to find the platform. During this time, the hidden platform was submerged in the center of one of the 4 quadrants and remained in that location for the entire duration of the experiment. For each trial, the rat was taken from the home cage and placed into the water maze at one of the four quasi-randomly determined locations with its head facing and almost touching the pool wall. Trial began when the rat was released by the experimenter and ended when it climbed to the top of the hidden platform. The mean escape latency was recorded. The maximum trial length was 60 s. If by that time, the rat was not able to locate the top of the platform, the trial was terminated. It was guided to the platform where it was allowed to stay for 20 s and then again the process of recording escape latency of another 60 s was started. The rat was removed from the platform and placed in the pool again to begin the next trial. Normally, the escape latency declines during acquisition trial from 60 s to around 15 s when the animal learns the location of the hidden platform.

In the global ischemic model, the animals went into 4 days training (4 trials each day) to learn the location of the hidden platform in MWM before induction of GCI. The experimental readings of escape latency were recorded on the 7th day after the induction of ischemia before the animal was sacrificed. The cutoff time was 60 s.

Assessment of gross behavioral activity (closed field activity)

Gross behavioral activity was assessed by digital actophotometer. Each animal was observed over a period of 5 min in a square (30 cm) closed arena equipped with infrared light-sensitive photocells and values expressed as counts per 5 min. The beams in actophotometer cut by the animal were taken as a measure of movements. The apparatus was placed in a darkened, light- and sound-attenuated, and ventilated testing room.

Gross behavioral activity was recorded on zero-day (previous day to the induction of ischemia) and then on the 7th day after the induction of ischemia and ICV administration.

Assessment of histological changes

The brains were rapidly removed and fixed by immersion in 10% formalin. Subsequently, they were embedded in paraffin wax, cut into 5-µm thick sections and stained with Hematoxylin and Eosin stain. Hippocampal brain sections were examined under bright field illumination (AHBT-51, Olympus Vanox Research Microscope, Japan). We analyzed different areas in each section of 5 µm thickness. To examine the cell damage, the neurons were seen on at least three sections at each level at a ×400 magnification by an investigator who was blind for the treatment of the animals. Both intact and damaged neurons were seen in at least three areas at increased magnification. The level of neuroprotection and neurodegeneration was based on the degree of neuronal loss.

RESULTS AND DISCUSSION

Effect of AICAR and 3-Br-7-NI on biochemical parameters in brain homogenate MDA levels

Ischemic and vehicle control groups were found to show a significant rise in MDA levels and reduction in GSH levels in brain as compared to the sham control animals. 4 days treatment of ischemic animals with AICAR (25, 50, and 100 µg/rat, ICV) was found to significantly exacerbate the ischemic damage and further elevate the level of MDA. Furthermore, 3-Br-7-NI (10 and 20 µg/rat, ICV) was found to significantly attenuate their elevated levels of MDA. The MDA levels in the brain homogenate of rat treated with AICAR 25 µg/rat were found to be significantly more than that of GCI control, and increased significantly with increasing the dose to 50 µg [Table 1]. Further, increasing the dose of AICAR to 100 µg leads to further increase in MDA level but not significantly more than that of 50 µg. While 3-Br-7-NI did not show any *per se* effect at both doses (10 and 20 µg/rat, ICV), but it decreased the levels of MDA significantly and dose-dependently as compared with GCI control. However, 3-Br-7-NI 20 µg/rat, ICV showed comparatively more significant effect than 10 µg of 3-Br-7-NI in attenuating the elevated MDA levels. Moreover, 3-Br-7-NI at doses 10 and 20 µg/rat show a significant and dose-dependent decrease in MDA levels when administered to ischemic rats treated with AICAR 50 µg. However, 3-Br-7-NI 20 µg/rat fails to show a significant decrease in MDA levels as compared to group GCI with AICAR (100 µg/rat, ICV). Hence, due to some controversial results in an animal with ischemia and AICAR100 µg/rat, we have selected the dose of AICAR 50 µg/rat, ICV appropriate for combination study.

Table 1: Effect of AICAR and 3-Br-7-NI on various oxidative stress parameters evaluated in brain in BCCAO model

Groups	LDH (% control)	AChE (nM/mg protein)
Sham	60.20±7.91	12.064±0.96
GCI	283.01±18.23[a]	7.75±0.45[a]
AICAR-25 µg	384.86±21.06[b]	5.94±0.28[b]
AICAR-50 µg	457.44±18.42[b,c]	4.52±0.37[b,c]
AICAR-100 µg	501.89±19.65[b,c]	3.79±0.37[b,c]
AICAR-50 µg (*per se*)	70.33±9.29	11.50±0.70
AICAR-100 µg (*per se*)	86.08±9.67	11.29±0.85[a]
NI-10 µg	195.24±8.98[b]	9.10±0.21[b]
NI-20 µg	91.15±10.12[b]	10.81±0.43[b]
NI-20 (*per se*)	65.43±7.49	12.01±0.67
AICAR-50+NI-10	232.38±20.00[b,d]	8.95±0.40[b,d]
AICAR-50+NI-20	78.38±8.22[b,d,e]	11.01±0.43[b,d,e]

Values are mean±SEM, statistical analysis one-way ANOVA followed by Tukey's test. [a]P<0.05 versus sham group; [b]P<0.05 versus ischemic control group; [c]P<0.05 versus AICAR-25; [d]P<0.05 versus AICAR-50; [e]P<0.05 versus AICAR-50+NI-10. SEM: Standard error mean, AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, BCCAO: Bilateral common carotid artery occlusion, GCI: Global cerebral ischemia, NI: Nitroindazole, AChE: Acetylcholinesterase, LDH: Lactate dehydrogenase

Reduced GSH levels

Ischemic and vehicle control groups were found to show a significant reduction in GSH levels in brain as compared to the sham control animals. 4 days treatment of ischemic animals with AICAR (25, 50, and 100 µg/rat, ICV) was found to significantly exacerbate the ischemic damage and further decrease the level of GSH [Table 2]. Furthermore, 3-Br-7-NI (10 and 20 µg/rat, ICV) was found to significantly restore the reduced levels of GSH in brains of ischemic animals. Dose of AICAR 25 µg/rat when administered with GCI treated rats was found to increase MDA levels significantly as compared to that of GCI control and observed to decrease significantly with increasing the dose to 50 µg. Further, increasing the dose of AICAR to 100 µg leads to further increase in MDA level but not significantly more than that of 50 µg. While 3-Br-7-NI did not show any effect in *per se* group (20 µg/rat, ICV), but it increased the levels of reduced GSH significantly and dose-dependently as compared with GCI control. However, 3-Br-7-NI 20 µg/rat, ICV showed comparatively more significant effect than 10 µg of 3-Br-7-NI in elevating the decreased GSH levels. Moreover, 3-Br-7-NI at doses 10 and 20 µg/rat show a significant and dose-dependent increase in GSH levels when administered to animals of GCI group and ischemic rats treated with AICAR 50 µg. However, 3-Br-7-NI 20 µg/rat fails to show a significant increase in GSH levels in ischemic animals treated with AICAR 100 µg/rat, ICV, as compared to GSH level in an ischemic animal with AICAR 100 µg/rat, ICV dose. Hence, due to some controversial results in an animal with ischemia and AICAR 100 µg/rat, we have selected the dose of AICAR 50 µg/rat, ICV appropriate for combination study.

Nitrite and LDH levels

In ischemic control groups, the levels of nitrite and LDH in brain were found to be significantly increased as compared with the sham control animals. Levels of nitrite [Table 1] and LDH [Table 1]. In ischemic animals were significantly increased. AICAR administration at the doses 25, 50, and 100 µg/rat, ICV significantly and dose-dependently increased the nitrite [Table 1] and LDH levels [Table 1] as compared to ischemic control rats. While elevated levels of nitrite and LDH in ischemic animals treated with AICAR 50 µg/rat ICV were significantly decreased by 3-Br-7-NI (10, 20 µg/rat, ICV) after treatment for 4 days. However, 3-Br-7-NI 20 µg/rat, ICV was found to be more effective than 3-Br-7-NI 10 µg/rat, ICV the effect of 50 and 100 µg/rat, ICV doses of AICAR were found to be approximately equivalent to each other regarding both the parameters, while significantly higher than GCI control and GCI with 25 µg/rat, ICV dose. 3-Br-7-NI 20 µg/rat, ICV shows a significant decrease on both the parameters when compared with GCI control and AICAR (25, 50, and 100 µg/rat, ICV) treated ischemic animals. *Per se* groups of AICAR (50 and 100 µg/rat, ICV) and 3-Br-7-NI 20 µg/rat, ICV displayed results similar to that of sham-control as expected.

AChE Levels

The levels of AChE were found to be significantly decreased in ischemic and vehicle control animals as compared to sham-control animals indicating cholinergic neuronal damage [Table 2]. Treatment of ischemic animals for 4 days with AICAR (25, 50, and 100 µg/rat, ICV) significantly and dose-dependently decreased the AChE as in comparison with GCI control animals [Table 2]. However, 3-Br-7-NI when administered at doses 10 and 20 µg/rat, ICV in animals of groups GCI, GCI with AICAR (25, 50, and 100 µg/rat) restored the AChE levels in brain. However, 3-Br-7-nitroindazole 20 µg/rat, ICV showed comparatively more significant effect than 10 µg.

DISCUSSION

GCI is a clinical condition that causes a deprivation of blood supply and energy in the brain due to blockade of carotid arteries.[2]The reduction in oxygen and glucose in cells causes disruption of protein synthesis, depletion of intracellular energy stores, destabilization of the cell membrane, opening of voltage-gated Ca^{2+} channels, and activation of the N-methyl-D-aspartic acid receptor. These conditions lead to excitotoxicity and oxidative damage. Stimulation of NOS by increasing Ca^{2+} levels causes accumulations of nitric oxide (NO), superoxide, peroxynitrite (ONOO), and free radicals, which further damage the cell membrane and may lead to DNA damage. In the attempt to repair damage and return neurons to homeostasis, numerous energy-consuming processes are activated. Overactivation of these pathways during ischemia can lead to complete energy failure and cell death. Most affected brain area as a consequence of GCI is CA1 neurons of the hippocampus.[27] Neuronal death in global ischemia proceeds 1st and 3rd day after the insult, and the process was referred to as delayed neuronal death.[9] Cerebral ischemia leads to cell death in CA1 region of the hippocampus, which occurs 3–4 days after an initial ischemic insult and neurodegeneration were evident by 3 days of reperfusion and neuronal death culminates by 6 days.[8] Since hippocampus was the region associated with learning and memory and regulation of locomotor activity, cerebral ischemia leads to severe biochemical alteration, histopathological damage, cognitive deficits, and hyperlocomotion.[28]

Table 2: Effect of AICAR and 3-Br-7-NI on various biochemical parameters evaluated in brain in BCCAO model

Groups	MDA (nM/mg protein)	GSH (µM/mg protein)	Nitrite/Nitrate (µM/mg protein)
Sham	0.466±0.09	0.154±0.005	0.560±0.05
GCI	2.33±0.16[a]	0.043±0.003[a]	4.26±0.09[a]
AICAR-25 µg	4.059±0.33[b]	0.031±0.004[b]	5.43±0.17[b]
AICAR-50 µg	5.05±0.19[b,c]	0.020±0.002[b,c]	6.61±0.16[b,c]
AICAR-100 µg	5.36±0.23[b,c]	0.019±0.003[b,c]	7.03±0.18[b,c]
AICAR-50 µg (*per se*)	0.47±0.09	0.147±0.004	0.66±0.10
AICAR-100 µg (*per se*)	1.18±0.11[a]	0.052±0.004[a]	1.48±0.11[a]
NI-10 µg	1.69±0.15[b]	0.120±0.004[b]	2.18±0.18[b]
NI-20 µg	0.58±0.05[b]	0.140±0.003[b]	1.12±0.12[b]
NI-20 (*per se*)	0.49±0.16	0.151±0.005	0.67±0.08
AICAR-50+NI-10	1.62±0.19[b,d]	0.118±0.005[b,d]	2.12±0.19[b,d]
AICAR-50+NI-20	0.82±0.06[b,d,e]	0.148±0.004[b,d,e]	0.64±0.14[b,d,e]

Values are mean±SEM, statistical analysis one-way ANOVA followed by Tukey's test. [a]$P<0.05$ versus sham group; [b]$P<0.05$ versus ischemic control group; [c]$P<0.05$ versus AICAR-25; [d]$P<0.05$ versus AICAR-50; [e]$P<0.05$ versus AICAR-50+NI-10. SEM: Standard error mean, AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, BCCAO: Bilateral common carotid artery occlusion, NI: Nitroindazole

To evaluate the effect of AMPK activation in cerebral ischemia, Wistar rats were used in the present study because rats were a close resemblance of the cerebrovascular anatomy and physiology to the higher species and were widely used in cerebral ischemia studies.[5] GCI was induced by bilateral carotid artery occlusion for 10 min. Cerebral ischemia leads to AMPK activation and further administration of AICAR leads to sustained activation of AMPK. Literature has suggested that AMPK activation causes stimulation of mitochondrial biogenesis resulting in an increase in free radical accumulation.[29] These free radicals further cause oxidative stress, therefore, affecting the oxidative stress parameters such as MDA levels, LDH levels, GSH levels, ONOO- level, and ONOO- further increase AChE levels in brain.

In the present study, cerebral ischemia was noted to cause: Increased MDA levels, decreased GSH levels, increased LDH levels, and decreased AChE levels, and increased nitrate levels in brain homogenate. Moreover, it was also been noted to produce an increased locomotor activity as well as increased latency period in MWM. AMPK activation using AICAR in an ischemic animal was noted to further exacerbate the ischemic damage that was assessed by biochemical as well as behavioral estimations in the present study.

AMPK activation through AICAR administration was observed to damage cortex and hippocampal regions of brain as are evident from photomicrographs [Figure 1 and Panel C-E]. Hippocampus is the region associated with learning, memory, and locomotion. Hence, damage to hippocampal region leads to a deficit in learning and memory as well as in increased locomotor activity. The damage to this region induced by AMPK activation was confirmed by an increase in latency period in MWM [Table 3], and hyperlocomotion in actophotometer [Table 3] of AICAR treated ischemic animals in the study. The photomicrographs of hippocampus show significant histopathological changes showing the neuronal damage which coincides with the increased locomotor activity and latency period as well as alteration in LDH, AChE [Table 1], and oxidative stress parameters [Table 2]. However, administration of nNOS inhibitor has shown that the neuroprotective role in GCI as well as AICAR treated ischemic rats as is evident from the photomicrographs [Figure 2 and Panel F-I].

In the present study, AMPK was activated using a well-known AMPK activator AICAR, and nNOS was inhibited by 3-bromo-7-NI. Therefore, it was found that GCI leads to increased oxidative stress that was accessed by estimation of biochemical parameters. AMPK is a heterotrimeric serine-threonine kinase composed of a catalytic-α, and 2 regulatory β, and γ-subunits. Studies have demonstrated the expression of AMPK in numerous tissues, but Gao *et al*. (1995) examined the brain-specific AMPK in detail.[30] Various studies have been reported that AMPK is expressed in brain in neurons[31] as well as in glial cells. AMPK catalytic activity is enhanced not only by AMP binding but also by α-subunit phosphorylation by an upstream kinase Ca^{2+}-calmodulin-dependent protein kinase kinase-beta. In addition to activation by hormones and change in ATP/AMP levels, AMPK is also activated by stresses such as hypoxia, ischemia, oxidative, and nitrosative stresses.

In contrast to the findings of Culmsee *et al.,* 2001, who have reported a neuroprotective role of AMPK in isolated hippocampal neurons under different experimental conditions and our results have suggested that the sustained activation of AMPK produces neurodegeneration as was evident from results. These results were consistent; Ramamurthy and Ronnet, 2006, who have also reported neurodegeneration with AMPK activation. AMPK activation in cerebral ischemia may cause neurodegeneration through any of the following pathways: Activation of JNK,[32] which further results in neurodegeneration by stimulating cytochrome-C, caspases (3/9), simulating mitochondrial biogenesis and so free radical accumulation,[33] and increased production of ONOO- possibly through nNOS activation.

Thus, it may be concluded from the present study that AMPK activation in ischemic animals leads to neurodegeneration through any of the above-mentioned pathways and possibly also through nNOS activation. As discussed previously the prolonged activation of nNOS results in accumulation of peroxynitrite, which further causes DNA damage and PARP activation an energy consumptive process, which further may contribute to neurodegeneration.

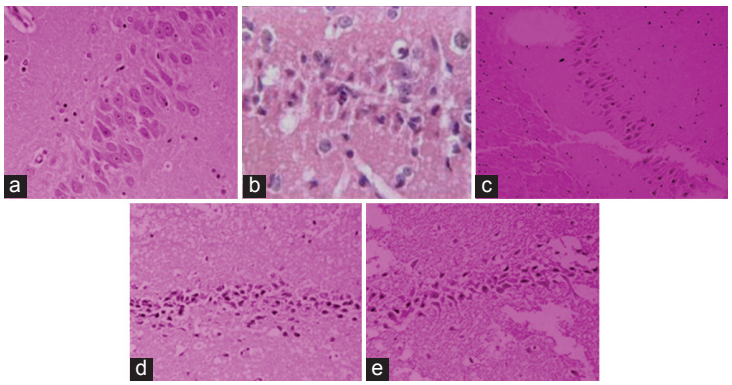

Figure 1: Panel a-i: Photomicrographs (×400) of Hematoxylin and Eosin stained brain hippocampal sections showing the effect of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and 3-Br-7-nitroindazole on cerebral ischemia-induced neurodegeneration. Panel a: Sham control; Panel b: Global cerebral ischemia (GCI) control showing hippocampal neurodegeneration characteristic of confidence interval: Panel c: AICAR 25 μg treated GCI; Panel d and e AICAR (50, 100 μg/rat, intracerebroventricularly) treated GCI showing further exacerbate the neurodegeneration

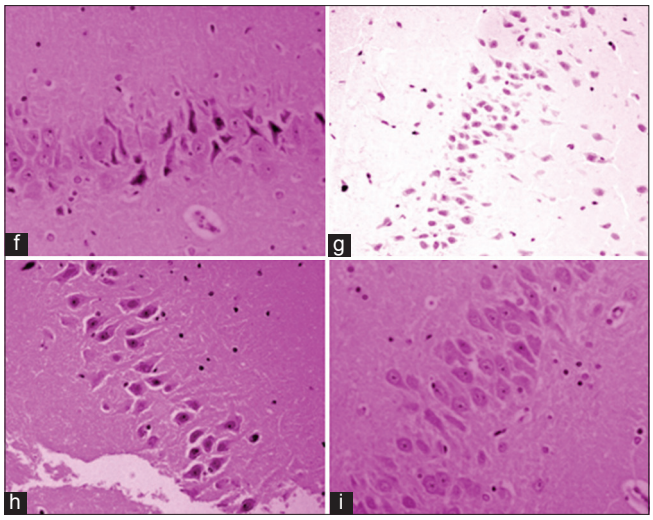

Figure 2: Panel a-i: Photomicrographs (×400) of Hematoxylin and Eosin stained brain hippocampal sections showing the effect of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and 3-Br-7-nitroindazole (NI) on cerebral ischemia-induced neurodegeneration. Panel f and g: NI (10, 20 µg/rat, intracerebroventricularly) treated global cerebral ischemia (GCI) show neuroprotection against GCI-induced neurodegeneration; Panel h: AICAR-50 µg and NI-10 µg treated GCI, NI-10 µg showing to decrease the level of neurodegeneration; Panel i: AICAR-50 µg and NI-20 µg treated GCI, NI-20 µg showing to markedly decrease the level of neurodegeneration

Table 3: Effect of AICAR and 3-Br-7-NI on various behavioral parameters evaluated in BCCAO model

Groups	Locomotor activity (counts per 5 min)	Latency period in Morris water maze (in sec.)
Sham	215.33±8.41	5.87±0.50
GCI	285.33±7.85[a]	162.66±3.5[a]
AICAR-25 µg	340.83±11.78[b]	69.33±2.94[b]
AICAR-50 µg	380.66±10.34[b,c]	82.33±5.56[b,c]
AICAR-100 µg	390.66±15.76[b,c]	84.16±2.19[b,c]
AICAR-50 µg (*per se*)	215.83±11.99	7.333±1.38
AICAR-100 µg (*per se*)	223.83±4.483	8.66±1.11
NI-10 µg	250.50±9.4[b]	30.83±8.28[b]
NI-20 µg	220.16±8.34[b]	7.66±2.73[b]
NI-20(*per se*)	224.16±6.38	6.66±1.02
AICAR-50+NI-10	279.16±8.34[b,d]	34.16±5.67[b,d]
AICAR-50+NI-20	222.33±11.54[b,d,e]	8.75±1.52[b,d,e]

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, BCCAO: Bilateral common carotid artery occlusion, GCI: Global cerebral ischemia, NI: Nitroindazole

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